

**ENERGY EXPENDITURE IN TYPE I AND TYPE II DIABETES MELLITUS:  
THE ROLE OF BROWN ADIPOSE TISSUE IN MAN**

by

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A dissertation submitted for the degree of

Doctor of Medicine  
to the  
University of Edinburgh

1987



# DECLARATION

I declare that the text of this dissertation submitted to the University of Edinburgh for the degree of Doctor of Medicine has been composed entirely by myself and is based on my own observations. The studies were planned, executed and analysed by myself, except as indicated.

February 1987

### ACKNOWLEDGEMENTS

The studies described in this thesis were performed while employed as a Clinical Research Fellow in the Department of Medicine, University of Dundee, during the period February 1984 to February 1986. Financial support from the Scottish Home and Health Department, the Medical Research Council and Nordisk UK is gratefully acknowledged. A grant from Tenovus (Scotland) provided the majority of insulin infusion pumps whose running costs were met by a grant from Tayside Health Board Research Committee.

The calorimeter suite was constructed with the help and advice of Mr R. Rimmer, Ninewells Hospital, from a design developed at the MRC Dunn Nutrition Unit, Cambridge, who also provided the computer programme for calculation of metabolic rate. Biochemical assays were performed with the expert technical assistance of Mrs Jean Baty. I also thank the nursing staff from the Diabetic Clinic for their help with review of the diabetic patients and special thanks to these subjects who devoted much time and effort to participate in the clinical studies.

The brown adipose tissue studies would not have been possible without the close cooperation of the surgeons - Mr J.P.A. Weaver, Mr K. Baxby, Mr A. Gunn and Mr P.E. Preece from the Dundee hospitals. I also wish to thank Mr A. Smith and Dr P. Padfield of the Western General Hospital, Edinburgh, and Dr J. Brown of the MRC Hypertension Unit, Glasgow, who provided me with specimens from patients with pheochromocytoma. Dr D. Rushton and the staff of the Post Mortem Room, Dundee Royal Infirmary were extremely generous in allowing unlimited access to dissect post mortem tissue. Dr D. Hopwood and staff, Ninewells Hospital and Professor J.D. Lever and Ms D. Symons, University College, Cardiff provided an unrivalled

technical expertise in the histological analysis of the specimens.

The brown adipose tissue mitochondrial and isolated adipocyte studies were performed in conjunction with Drs Sonia Cunningham and David Nicholls whose patience and biochemical expertise enabled these studies, which involved working within the strict limitations of an extremely finite source of human tissue, to be performed.

For statistical advice I thank Mr S. Ogston and Dr I. Crombie and I am indebted to numerous friends and colleagues who kept my flagging spirits going during the execution of the studies and writing of the thesis.

The enthusiasm of Dr Ray Newton first kindled my interest in diabetes and introduced me to my mentor and source of constant support and discussion, Dr Roland Jung, to whom my special thanks are due.

Finally I thank Maureen Hughes for her expert typing and for never flinching at the sight of another draft.



List of Abbreviations used in the Text

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
$\beta$	Beta
BAT	Brown adipose tissue
BDH	British Drug House
BMI	Body mass index
CA	Cold adapted
CO <sub>2</sub>	Carbon dioxide
CoA	Coenzyme A
COX	Cytochrome oxidase
CSII	Continuous subcutaneous insulin infusion
CT	Conventional therapy
CV	Coefficient of variation
D	Dalton
EDTA	Ethylene diamine tetra acetic acid
EGTA	Ethylene glycol tetra acetic acid
F	Female
GDP	Guanosine diphosphate
HbA <sub>1</sub>	Haemoglobin A <sub>1</sub>
H and E	Haemotoxylin and eosin
<sup>125</sup> I	Iodine-125
IBW	Ideal body weight
M	Male
N <sub>2</sub>	Nitrogen
NAD	Nicotinamide adenosine dinucleotide
NADH	Nicotinamide adenosine dinucleotide (reduced)

O <sub>2</sub>	Oxygen
P	Phosphate
PEG	Polyethylene glycol
PVC	Polyvinyl chloride
RIA	Radioimmunoassay
RMR	Resting metabolic rate
RPM	Revolutions per minute
RQ	Respiratory quotient
SD	Standard deviation
SDA	Specific dynamic action
SEM	Standard error of the mean
T <sub>3</sub>	Triiodothyronine
T <sub>4</sub>	Tetraiodothyronine
TSH	Thyroid stimulating hormone
VO <sub>2</sub>	Oxygen consumption
WA	Warm adapted

UnitsWeight

µg	- microgramme
mg	- milligramme
g	- gramme
kg	- kilogramme

Time

min	- minute
yr	- years

Length

Å	- Angstrom
nm	- nanometre
µm	- micrometre
cm	- centimetre
m	- metres

Concentration

nmol/l	- nanomoles per litre
µmol/l	- micromoles per litre
mmol/l	- millimoles per litre
M	- molar
mu/l	- milliunits per litre
u	- units

Energy

kJ	- kilojoules
MJ	- megajoule
kcal	- kilocalorie

Conversion : kJ to kcal - 1 kJ = 0.24 kcal.

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## ABSTRACT

**INTRODUCTION:** Abnormalities of energy expenditure (EE) in obesity and type II diabetes mellitus have been described in animal models and adult man and it has been suggested that these abnormalities predispose to weight gain. In rodents there is evidence for a requirement for insulin in regulatory thermogenesis mediated via brown adipose tissue (BAT).

The hypotheses that, firstly, insulin has a role in the control of EE and, secondly, that this is mediated by BAT in adult man were tested in the present studies.

**METHODS:** Three components of daily EE: resting metabolic rate (RMR), and the thermic responses to a mixed liquid meal (32.6 KJ/kg) and to an infusion of noradrenaline (NA), 0.1 µg/kg/min, were measured using indirect calorimetry in type I and type II diabetic subjects.

The components of EE were measured in 9 lean type I diabetics initially while poorly controlled on conventional therapy (CT), HbA<sub>1c</sub> 12.1±0.7% (mean±SEM), and then when optimally controlled on continuous subcutaneous insulin infusion (CSII) (HbA<sub>1c</sub> 7.5±0.2%). The response to fat supplementation for one week (5.23 MJ/day) was assessed and the results compared to 8 non-diabetic control subjects.

These parameters were also measured in 8 type II diabetics controlled on diet and oral hypoglycaemic agents ( $\text{HbA}_1$   $8.6 \pm 0.7\%$ ) in order to ascertain whether the alterations in body weight during metformin therapy (weight loss) or sulphonylurea therapy (weight gain) were reflected in alterations of EE.

An estimation of the thermogenic potential of BAT in adult man was attempted by histological (light and electron microscopy) and biochemical (GDP binding, cytochrome c oxidase activity and respiration of isolated mitochondria and adipocytes) studies on perirenal BAT obtained at post mortem and during surgery.

## RESULTS:

1. Poorly controlled type I diabetic subjects had a significantly ( $p < .01$ ) raised RMR ( $4.92 \pm 0.27$  KJ/min) which returned to predicted value ( $4.6 \pm 0.34$  KJ/min) on attainment of optimal glycaemic control. This decrease in RMR may partly explain the mean gain in weight (3.5 kg) observed during CSII. The thermic response to infused NA was decreased by over 50% during CT ( $27.1 \pm 3.4$  KJ controls vs.  $11.1 \pm 3.7$  KJ diabetic CT;  $p < .01$ ) and did not significantly improve during CSII ( $13.4 \pm 3.7$  KJ). A blunted thermic response to a meal was observed during fat supplementation with CT ( $44.8 \pm 14.0$  KJ) but CSII corrected this ( $71 \pm 9.9$  KJ). Hence insulin has a role in the control of EE but precise replacement does not correct all these abnormalities in Type I diabetes mellitus.



2. Type II diabetic subjects had a normal RMR and thermic responses and these were similar whether on metformin or sulphonylurea therapy. This suggests that alterations in EE do not account for the changes in weight during treatment with these agents.
3. Histological and biochemical studies demonstrated the presence of functional BAT in adult man with activity equivalent to the partially cold-adapted guinea pig suggesting some potential for thermogenesis in adult man. However, it was calculated that this tissue can account for only 0.2% of the rise in oxygen consumption during NA infusion and suggests that this tissue has little energetic significance in adult man.

CONCLUSIONS: These data suggest that although type I diabetes mellitus may be associated with abnormalities of EE these are unlikely to be mediated through BAT metabolism in man.

## PREFACE

"You will see one man fat who eats moderately well and another lean who eats a great deal".

- James Boswell, 1782

This apparent paradox observed by that irascible Scot, James Boswell, remains as hotly debated a topic amongst the lay and scientific population in the present day as it did over two hundred years ago.

Obesity is now a major public health problem in the Western world and can be considered a multisystem disorder responsible for an excess of mortality and morbidity in over one-fifth of the adult population. Apart from the association of obesity with type II diabetes mellitus and cardiovascular disease; gallbladder disease, an excess of carcinomas of the large bowel and prostate, osteoarthritis and respiratory problems are well recognised complications of this disorder (Royal College of Physicians, 1983).

Yet, although it is universally agreed that obesity must result from an imbalance between energy intake and expenditure, there is still no clear understanding of the influence of each of these parameters in the aetiology of obesity in man. Studies which have attempted to examine the role of energy intake have failed to demonstrate a conclusive link between hyperphagia and obesity (Garrow, 1978). One major epidemiological survey involving over 3,400 subjects reported the unexpected finding of negative correlations between energy intake and obesity and energy intake and glucose intolerance (Keen et. al., 1979). Methodological problems may partly explain these paradoxical results as one of the major obstacles to the accurate measurement of nutrient intake has been that the caloric intake of

free-living subjects, under the influence of the social and environmental factors that dictate what type and how much food we eat, is probably very different from intakes recorded under the different pressures of taking part in a scientific dietary survey. A recent report has suggested that errors in assessment of dietary intake may approach 500 kcal/day (Prentice et. al., 1986).

However, the alternative explanation for these findings, which has increasingly been investigated recently, is that a diminution of energy expenditure may contribute to the development of obesity. This school of thought is supported by evidence from animal models in which dissipation of excess energy through brown adipose tissue metabolism results in the maintenance of body weight during dietary excess (Rothwell and Stock, 1979) and conversely abnormalities of this tissue appear to predispose to weight gain in certain genetically obese strains of mice (Trayhurn et. al., 1977).

Abnormalities of thermogenesis have been demonstrated in some obese subjects (Jung et. al., 1979; Shetty et. al., 1981) and it has been suggested that these abnormalities may predispose to the obese state by limiting energy expenditure in man (James and Trayhurn, 1981).

More recently, insulin resistance has been demonstrated to limit energy expenditure following intravenous (Ravussin et. al., 1985) and oral glucose (Golay et. al., 1982) in obese and type II diabetic subjects and it has been further suggested that insulin may have a key role in controlling energy expenditure in man (Felig, 1984).

Animal research has supported this concept as insulin appears to have a permissive role in the expression of brown adipose tissue thermogenesis (Seydoux et. al., 1983) and the ability to increase energy expenditure in response to overfeeding is diminished when rats are rendered diabetic and is

corrected by insulin replacement (Rothwell and Stock, 1981).

Thus considerable evidence has accumulated implicating insulin in the control of energy balance and the aims of the present studies were to examine further aspects of energy expenditure in diabetic and non-diabetic man.

In Chapter I, the evidence for the thermogenic hypothesis and its application to animal models of obesity and lean, obese and diabetic man will be reviewed.

Following a description of the methodology, results of experiments designed to examine the role of insulin in modulating energy balance outwith the context of obesity will be presented by studying the effect of glycaemic control on energy expenditure in lean type I diabetic subjects.

Weight control is a major problem in the management of the type II diabetic subject on oral glycaemic therapy. Experiments were therefore designed to determine whether the documented weight loss on metformin therapy and the tendency to gain weight on sulphonylurea derivatives (Clarke and Duncan, 1968) are related to alterations of energy expenditure in these subjects.

The thermogenic hypothesis of obesity in animals and its extrapolation to adult man hinges on the central role of brown adipose tissue in the control of energy expenditure. In the recommendations of the Royal College of Physicians Report on Obesity (1983) it was noted

"no hormonal basis for the metabolic susceptibility to weight gain has yet been found. More work is needed before possible mechanisms . . . e.g., brown adipose tissue metabolism can be defined and shown to be quantitatively important in determining weight gain"

- (Recommendation No. 6).

The final aim of the present studies was to attempt to determine the functional capacity of this tissue in adult man.

## Chapter I

### The Thermogenic Hypothesis

## SUMMARY

Obesity is a major clinical problem and it is well documented that a proportion of obese subjects develop clinical diabetes and that many diabetic subjects are overweight.

Animal studies have indicated the importance of thermogenesis in the maintenance of a stable weight during overfeeding and that abnormalities of this homeostatic mechanism may result in obesity. In small rodents regulatory thermogenesis is mediated via brown adipose tissue metabolism and evidence suggests that insulin may have a role in the hormonal control of the activity of this tissue.

Some, but not all, studies in obese and type II diabetic man have also demonstrated abnormalities of thermogenesis and it has been suggested that these abnormalities may predispose to the development of obesity by limiting energy expenditure. It has also been suggested that glucose intolerance and/or frank diabetes may further exacerbate this limitation of energy expenditure. The principal site of regulatory thermogenesis in man has, by extrapolation, been presumed to reside in brown adipose tissue; however there is little evidence to confirm or refute this hypothesis.

This introductory chapter summarises the results of studies performed in animals and man which have addressed the thermogenic hypothesis of obesity and provides the background to the aims of the present studies which were to examine further the role of glucose tolerance in the control of energy expenditure in lean non-diabetic and type I and type II diabetic man and to estimate the contribution of brown adipose tissue to thermogenesis in adult man.

### 1.1. INTRODUCTION

The thermogenic hypothesis dates back to the beginning of this century when the German physiologist, Neumann (1902), coined the term "lukuskonsumption" (literally, energy consumption) in an attempt to account for the relative constancy of his own body weight despite fluctuations in energy intake of over 30% during a three year period. The hypothesis postulates the existence of a homeostatic mechanism in man responsible for the maintenance of a stable body weight despite alterations in energy intake. Thus, when food is available ad libitum energetically wasteful (or thermogenic) mechanisms would be initiated to dissipate excess calories and prevent weight gain; conversely during periods of limited food availability these mechanisms would be inhibited to promote storage of energy. An extension of this argument would predict that subjects in whom this complex physiological system was disturbed, would demonstrate abnormalities of energy expenditure which could predispose to weight gain.

Before discussing the evidence for the existence of such a mechanism in animal models and man it is necessary to describe the various components that contribute to total daily energy expenditure in man.

### 1.2. COMPONENTS OF ENERGY EXPENDITURE

Energy expenditure is measured as the heat generated by the body either directly by the measurement of heat loss or indirectly by the calculation of heat production resulting from substrate oxidation.

To study the role of energy output in the regulation of body weight it is useful to divide daily energy expenditure into three separate components - the basal metabolic rate, thermogenesis and physical activity (Fig. 1.1(a)).

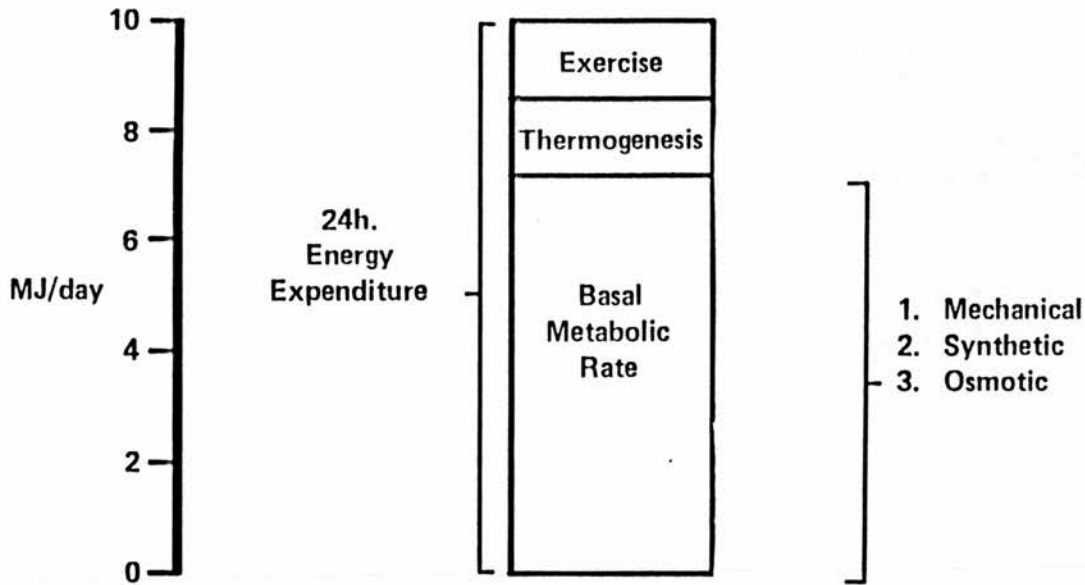


Fig. 1.1(a) COMPONENTS OF ENERGY EXPENDITURE

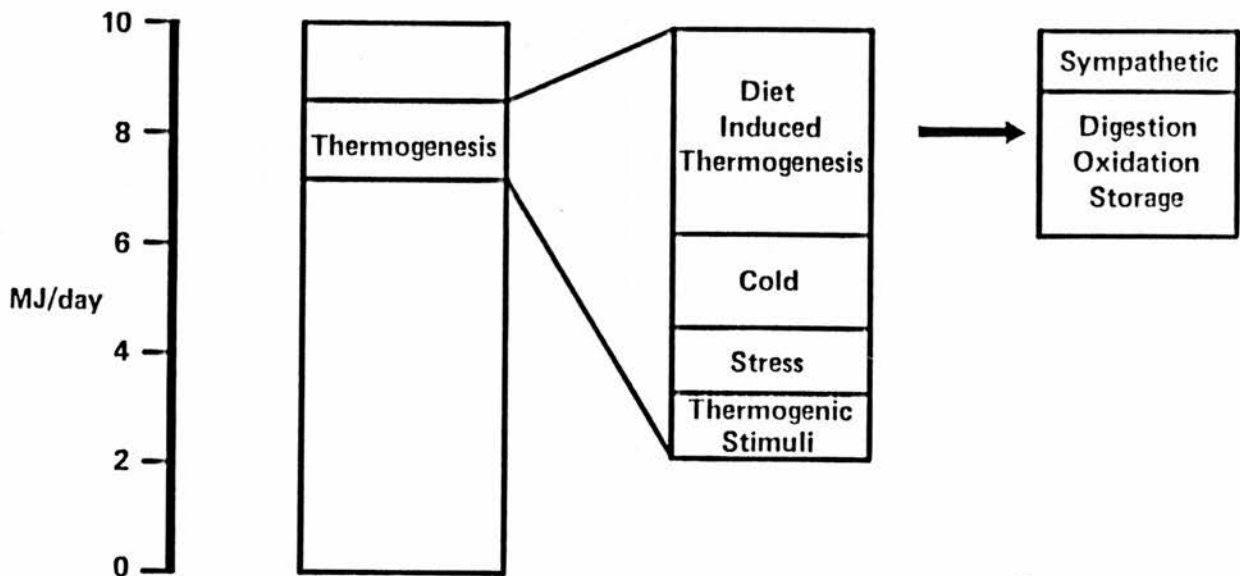


Fig. 1.1(b) THERMOGENESIS

Fig. 1.1. Energy Expenditure and Thermogenesis

- (a) Components of energy expenditure  
(b) Components of thermogenesis



### 1.2.1. Basal Metabolic Rate (Definition : Section 2.1.3)

Basal metabolic rate accounts for approximately 70% of total daily energy expenditure and is related to age, sex, fat free mass and thyroid status of the individual (Jequier, 1984). It represents the sum of energy expended to maintain mechanical processes such as resting muscle tone and myocardial contraction; osmotic pumps and the synthesis and breakdown of the major substrates - protein, carbohydrate and fat.

### 1.2.2. Thermogenesis (Fig. 1.1.(b))

Thermogenesis is defined as the energy expenditure above the basal metabolic rate in the resting state and includes the effects of food intake, cold exposure, stress and thermogenic agents such as caffeine (Jung et. al., 1981), ephedrine (Astrup et. al., 1985(a)) and cigarette smoking (Hofstetter et. al., 1986).

#### 1.2.2.1. Diet induced thermogenesis

Among the factors that stimulate thermogenesis in man, food intake plays a major role. Many expressions have been used to describe this phenomenon including specific dynamic action (SDA), postprandial thermogenesis and diet induced thermogenesis; the latter being the most widely employed nowadays. Diet induced thermogenesis consists of two components. The major component is the energy cost of digestion, absorption and storage of ingested nutrients - these processes are known collectively as "obligatory thermogenesis". However, for example, when glucose is infused the observed glucose induced thermogenesis is in excess of the calculated energy cost of absorption, oxidation and glycogen and lipid synthesis (Thiebaud et. al., 1983). This component of diet induced thermogenesis which cannot be accounted for by obligatory thermogenesis has been called

"facultative thermogenesis" (Acheson et. al., 1984). In small mammals there is considerable evidence that this latter component is expressed via brown adipose tissue (BAT) under the control of the sympathetic nervous system (Landsberg and Young, 1983).

In man there is evidence of sympathetic mediation of facultative thermogenesis as glucose induced thermogenesis can be partially suppressed with  $\beta$ -blockade (Acheson et. al., 1984). It is this component of diet induced thermogenesis that has been speculated to have a role in the adaptive response to overfeeding in man (Jequier, 1984).

#### 1.2.2.2. Cold induced thermogenesis

The metabolic response to cold exposure consists of two components: shivering thermogenesis which occurs as a consequence of muscular contraction and non-shivering thermogenesis which results from biochemical reactions not coupled to muscular activity.

Non-shivering thermogenesis in rats is largely attributable to brown adipose tissue activity and can be mimicked by injection of noradrenaline (Foster and Frydman, 1978(a)).

Human subjects also demonstrate non-shivering thermogenesis which is easily demonstrable in the neonate (Hey, 1975).

In the adult, cold exposure during curarisation, which inhibits muscular contraction, results in an increase in oxygen consumption (Jessen et. al., 1980) and noradrenaline infusion results in similar increases in energy expenditure (Jung et. al., 1979; Joy, 1963). Noradrenaline infusion has thus been employed as the pharmacological model for non-shivering thermogenesis in man.

### **1.2.3. Physical Exercise**

In most adults physical activity accounts for up to 20% of total daily expenditure (Fig. 1.1(a)). The energy cost of daily activity is difficult to assess and varies considerably both between individuals and in the same individual over a period of time. There is no evidence, as yet, to suggest that some feature of this component predisposes to the obese state although it remains a major adjunct to therapy in the management of the overweight subject.

## **1.3. THERMOGENESIS IN SMALL MAMMALS**

### **1.3.1. Non-shivering Thermogenesis**

Over 50 years following Neumann's original concept of "luxuskonsumption" (Neumann, 1902) the first preliminary evidence for abnormalities of energy expenditure predisposing to the development of the obese state was presented by Davis and Meyer (1954) who demonstrated defective thermoregulation in the genetically obese (ob/ob) mouse. They observed that these obese mutants died rapidly of hypothermia when exposed to an ambient temperature of 4°C in contrast to the survival of their lean littermates. This work was later confirmed and extended by Trayhurn and colleagues who demonstrated a diminished ability of the ob/ob mouse to initiate non-shivering thermogenesis both at low environmental temperature and in response to noradrenaline (Trayhurn and James, 1978). Further, this reduction in energy expenditure occurred prior to the development of hyperphagia in these animals (Trayhurn et. al., 1977) and could result in increased metabolic efficiency and weight gain.

Fig. 1.2 (overleaf)

During this period the contribution of BAT to the metabolic response to cold and noradrenaline in the newborn mammal such as the lamb (Alexander et. al., 1973), rabbit (Dawkins and Hull, 1964) and the human neonate (Aherne and Hull, 1966) was well recognised but its role in thermogenesis in the adult mammal was less well established. The relatively small amount of BAT present in the adult mammal (less than 1-2% body weight) and blood flow studies performed at this time suggested that this tissue did not have a major role in adult thermogenesis (Jansky and Hart, 1968).

Three major mechanisms to account for the cellular dissipation of heat through wasteful hydrolysis of ATP had been proposed.

The most clearly established mechanism was shivering itself in which ATP is utilised to provide energy for the random contractions of the acto-myosin complex (Fig. 1.2(a)) but obviously this cannot account for the non-shivering mechanisms.

Substrate cycling (Newsholme and Crabtree, 1976) occurs when two intermediates can be interconverted by two independent pathways one of which requires ATP which is not replenished in the reverse reaction. A classic example of this is the interconversion of fructose-6-phosphate to fructose-1, 6-bisphosphate in the glycolytic sequence (Fig. 1.2(b)) which contributes to the maintenance of thorax temperature in the flight muscle of the Bombus bumble bee. The triglyceride fatty acid cycle (Fig. 1.2(c)) is a similarly wasteful cycle but has recently been calculated to account for the dissipation of less than 10 kcal/day in man (Hammond and Johnston, 1987).

The final type of ATP hydrolytic mechanism which has been proposed relies upon Na-cycling at the plasma membrane and subsequent hydrolysis of

ATP by  $\text{Na}^+ - \text{K}^+$  ATPase. Although this mechanism may contribute towards thyroid induced thermogenesis (Smith and Edelman, 1979) evidence for this as a major thermogenic mechanism is lacking (Nicholls and Locke, 1983).

The major breakthrough in our understanding of mammalian thermogenesis occurred when Foster and Frydman (1978a) demonstrated that non-shivering thermogenesis in adult rats, as in younger animals, was in fact due primarily to BAT. Using a radiolabelled microsphere technique to calculate blood flow to different organs they showed that cold adapted rats infused with noradrenaline had an increase in blood flow to brown adipose tissue of about 31% of the cardiac output, while the increased flow to skeletal muscle was only in the region of 5% of the cardiac output. They calculated from arteriovenous oxygen uptake across interscapular brown adipose tissue that this tissue accounted for at least 60% of the calorogenic response of the cold adapted rat to infused noradrenaline, while skeletal muscle could not have been responsible for more than 12% of the increased heat production. They also demonstrated that the previous estimates of blood flow to BAT using  $^{86}\text{Rb}^+$  were erroneous as this technique overestimates when blood flow rates are low and underestimates when high (Foster and Frydman, 1978b).

Thus, the major effector of non-shivering thermogenesis in small mammals appeared to be the relatively small tissue mass of BAT.

### **1.3.2. Diet Induced Thermogenesis**

In 1979 Rothwell and Stock made the important observation that adult rats could become effectively cold-adapted simply by overfeeding. They performed a series of experiments in which normal rats were offered various palatable food items such as chocolates and crisps in addition to the normal stock diet. This "cafeteria feeding", as it became known, resulted in an

increase in energy intake of 80% yet the small weight gain that occurred accounted for only 8% of the excess energy consumed (Rothwell and Stock, 1979). A significant proportion of the remainder had been dissipated as heat as demonstrated by a 30% increase in resting oxygen consumption. The increase in thermogenesis could be abolished by propranolol indicating beta( $\beta$ )-mediated sympathetic activation. The cafeteria rats also demonstrated a 70% increase in oxygen consumption in response to the injection of noradrenaline compared to 6% in controls and this was associated with hyperplasia of brown adipose tissue.

### **1.3.3. The Cellular Mechanism of Brown Adipose Tissue Metabolism**

#### **1.3.3.1. Anatomy**

In mammals brown adipose tissue is located in relatively small deposits in a number of sites, in particular, in the interscapular region and along the great vessels of the thorax and abdomen (Dawkins and Hull, 1965). The structure and function of brown adipose tissue differs markedly from those of white adipose tissue. Each brown adipocyte possesses multilocular fat droplets in association with numerous mitochondria with dense cristae (Nedergaard and Lindberg, 1982). In contrast, the storage role of the white adipocyte is facilitated by a large unilocular fat droplet and its relative metabolic inactivity is reflected in a paucity of mitochondria.

Brown adipose tissue has an extensive capillary vascular network and its metabolism is regulated by rich sympathetic innervation (Girardier, 1983) in response to the major stimuli that promote thermogenesis - cold and food ingestion. The subsequent  $\beta$ -adrenergic stimulation initiates cyclic-AMP dependent activation of triglyceride lipase. The resultant fate of released non-esterified fatty acids is however not to provide an energy source for

Fig. 1.3 (overleaf)



distant organs as in white adipose tissue, but to act as a messenger to uncouple oxidative phosphorylation.

#### **1.3.3.2. Uncoupled oxidative phosphorylation**

The uncoupling hypothesis of brown adipose tissue thermogenesis was proposed by Nicholls (1976) and is schematically represented in Fig. 1.3. According to the chemiosmotic theory of mitochondrial substrate oxidative phosphorylation the flow of protons from the matrix across the inner mitochondrial membrane and their reentry back across this membrane is linked to the generation of ATP from ADP (Fig. 1.3, Step 5). Nicholls demonstrated the existence of a secondary pathway in brown adipose tissue mitochondria which facilitated the return of protons independent of ATP synthesis thus allowing substrate oxidation without ATP production (Nicholls and Locke, 1983).

This proton conductance pathway, which appears to be unique to brown adipose tissue mitochondria, is inhibited by purine nucleotides in particular guanosine diphosphate (GDP) and adenosine diphosphate (ADP). The nucleotides bind to a specific 32,000 Dalton protein located on the inner mitochondrial membrane. The competitive binding of long chain fatty acyl CoA molecules displace the purine nucleotides and allows the leakage of protons uncoupled to ATP synthesis.

The acute sequence of events would be as follows: noradrenaline interacts with the  $\beta$ -receptor on the plasma membrane activating adenyl cyclase and cyclic AMP production (Fig. 1.3, Step 1). Activation of triglyceride lipase results in the production of non-esterified fatty acids and acyl CoA (Fig. 1.3, Step 2). Acyl CoA then displaces purine nucleotides at the inner mitochondrial membrane (Fig. 1.3, Step 3) thus resulting in functional uncoupling and the production of heat from fatty acid oxidation

without ATP synthesis (Fig. 1.3, Step 4).

#### **1.3.3.3. Control of Brown Adipose Tissue Metabolism**

Chronic adaptation to cold and overfeeding is reflected in the concentration of the 32,000 Dalton protein, thus the increased thermogenic capacity of this state is associated with hyperplasia of BAT, increase in the mitochondrial number (Buckowiecki et. al., 1982) and the concentration of the 32,000 Dalton protein in the inner mitochondrial membrane (Desautels et. al., 1978; Brooks et. al., 1980). This process appears to be under the control of the ambient sympathetic tone as this trophic response can be almost entirely abolished by pharmacological sympathectomy (Mory et. al., 1982) and mimicked by the presence of the catecholamine secreting tumour pheochromocytoma (Ricquier et. al., 1983) and chronic noradrenaline infusion (Mory et. al., 1984). Thyroid, pituitary and adrenocortical hormones appear to have only a permissive effect (Himms-Hagen, 1984).

#### **1.3.3.4. Insulin and Brown Adipose Tissue Metabolism**

Abnormalities of both non-shivering and diet-induced thermogenesis have been reported in association with animal models of diabetes mellitus. Rothwell and Stock (1981) demonstrated that rats rendered diabetic following the administration of streptozotocin failed to exhibit diet induced thermogenesis in response to cafeteria feeding and had blunted responses to the injection of noradrenaline both on normal stock-fed diet as well as during cafeteria feeding. Insulin replacement restored these responses to normal. They also demonstrated the failure of diabetic rats to maintain body temperature when exposed to cold (5°C) and confirmed the findings of Drury (1957) that these rats cannot survive cold environments unless replaced with

insulin.

Seydoux et. al., (1983) studied interscapular brown adipose tissue from streptozotocin diabetic rats and demonstrated atrophy of the fat pad, a diminished DNA and protein content, diminished basal and noradrenaline stimulated heat production and a reduced capacity for fatty acid oxidation. This group (Seydoux et. al., 1984) also demonstrated that indices of the thermogenic state (GDP binding and 32,000-MR protein) were decreased in streptozotocin diabetic rats and markedly increased following chronic infusion of insulin. Further evidence of the involvement of insulin in the genesis of energy dissipation in response to overfeeding was provided by Cunningham et. al., (1983). They made the important observation that in rats who became glucose intolerant during cafeteria feeding efficiency of weight gain was 18% higher than those rats with normal glucose tolerance. This was correlated with a failure of brown adipose tissue to increase in cellularity in response to cafeteria feeding and from these data they hypothesised that the link between diabetes and obesity may be bidirectional: obesity increasing the risk of development of diabetes, and impaired glucose tolerance accelerating the development of obesity by enhancing efficiency of weight gain.

Genetic models of obesity such as the ob/ob and db/db mouse also demonstrate abnormalities of thermogenesis in association with impaired glucose tolerance (Trayhurn and Fuller, 1980) and these factors may further compromise the already predisposed animal.

#### **1.3.3.5. Summary - Brown Adipose Tissue Metabolism in Small Rodents**

From this understanding of BAT physiology, cold and diet induced thermogenesis in normal rodents and the abnormalities of this process in the obese (ob/ob) mouse can now be explained in terms of brown adipose tissue

metabolism.

Sympathetic stimulation in the normal rat in response to cold or food results acutely in the uncoupling of mitochondrial oxidative phosphorylation and energy dissipation in the form of heat. The chronic adaptive response to cold or overfeeding results in the synthesis of uncoupling protein thus increasing the thermogenic potential of BAT.

Low concentrations of uncoupling protein (measured by the binding of radiolabelled GDP) account for the inability of the ob/ob mouse to increase heat production in response to sympathetic stimulation and burn off the excess energy consumed and thus it tends to gain weight (Brooks et. al., 1980).

#### **1.4. THERMOGENESIS IN ADULT MAN**

From these animal models, research in the field of control of energy balance and obesity in man became focussed in two areas.

Firstly the identification of a regulatory mechanism which could "buffer" weight gain during overfeeding and secondly the presence or absence of abnormalities of sympathetic or diet induced thermogenesis in obese subjects.

##### **1.4.1. Evidence from experimental overfeeding**

Anecdotal evidence for the presence of a homeostatic mechanism which would maintain body weight despite fluctuations in energy intake abounds in non-scientific and early scientific literature alike (Neumann, 1902; Gulick, 1922). However, it was not until the 1960s that experimental overfeeding confirmed this observation by demonstrating that a significant proportion of the excess energy intake could not be accounted for by the subsequent weight gain (Miller and Mumford, 1967; Durnin and Norgan,

1969). In one of the longest term overfeeding studies performed in the Vermont prisoners some individuals who normally maintained their weight on 12.6 MJ/day (3000 kcals), after several months of overfeeding required 23.8 MJ/day (5700 kcals) to maintain only slightly heavier weights (Sims, 1976). This increase in energy consumption was at least 40% in excess of the requirement to maintain the rise in RMR due to increased body weight and demonstrates that appreciable changes in food intake can be tolerated with only small changes in body weight (Himms-Hagan, 1984).

Thus, although some studies have failed to find any increase in energy expenditure during overfeeding (Glick et. al., 1977) the consensus of the literature points to an energy dissipative process occurring during overfeeding in man which some have construed as being analogous to the adaptive thermogenesis observed by Rothwell and Stock (1979) in the cafeteria fed rat (James and Trayhurn, 1981).

#### **1.4.2. Evidence of altered thermogenesis in obese states in man**

As there is no evidence for a decrease in the RMR predisposing obese individuals to weight gain (Ravussin et. al., 1982; Felig et. al., 1983; Welle et. al., 1984) attention has been focussed on those stimuli that increase energy expenditure above basal - the thermogenic stimuli of exercise, food, temperature and stress - in an attempt to identify whether differences in these parameters can account for a tendency to weight gain in obesity.

Studies of the effects of exercise and stress in lean and obese individuals are notoriously difficult to perform reproducibly and thus to interpret (Garrow, 1978) and will not be discussed further, although the sympathetic component of these processes has been studied using noradrenaline infusion as a pharmacological model.

Cold adaptation has been studied by examining the thermic responses not only to low environmental temperature but also to infused noradrenaline as a measure of non-shivering thermogenesis analogous to that in animal models.

Diet induced thermogenesis has been measured either as the thermic response to individual nutrients such as carbohydrate, fat and protein or as the thermic response to a meal.

#### **1.4.2.1. Non-shivering thermogenesis**

It is now generally accepted that non-shivering thermogenesis is an important homeostatic mechanism in neonates to maintain body temperature (Hey, 1975) and contributes to the increase in heat production in adult man during acute cold exposure (Dauncey, 1981).

There is also evidence of an adaptive response in non-shivering thermogenesis during prolonged cold exposure, which has been measured as an enhancement of the thermic effect of infused noradrenaline (Joy, 1963; Itoh, 1975). Differences in cold induced thermogenesis between lean and obese subjects have been reported (Blaza and Garrow, 1983), which have suggested that those with a propensity for obesity have a reduced drive for non-shivering thermogenesis similar to that in genetically obese rodents.

Using noradrenaline as a measure of non-shivering thermogenesis, blunted thermic responses to infusion of this catecholamine have been demonstrated in obese and post obese subjects suggesting that this defect was an inherent abnormality associated with the obese state (Jung et. al., 1979). However, other groups have failed to demonstrate such an abnormality in obese subjects (Katzeff et. al., 1986, Finer et. al., 1985).

Table 1.1 (overleaf)

#### **1.4.2.2. Diet induced thermogenesis**

A similar division of opinion has appeared in the literature regarding diet induced thermogenesis in lean and obese subjects. A summary of the results of 15 studies that have examined this parameter is shown in Table 1.1. These conflicting findings may be partly explained by differences in the caloric value of the meal, the length of time of study after ingestion, the selection of controls, and the heterogeneity of the obese population.

#### **1.4.3. Thermogenesis in diabetic man**

Historically, the measurement of oxygen consumption in diabetic subjects is not new.

Allen and Dubois (1916) studying the effect of starvation and an oatmeal diet on Type I diabetes, disputed Benedict and Joslin's (1910) previous findings of a 15-20% elevation of oxygen consumption and concluded that "the increase in basal metabolism in diabetes is generally absent or slight".

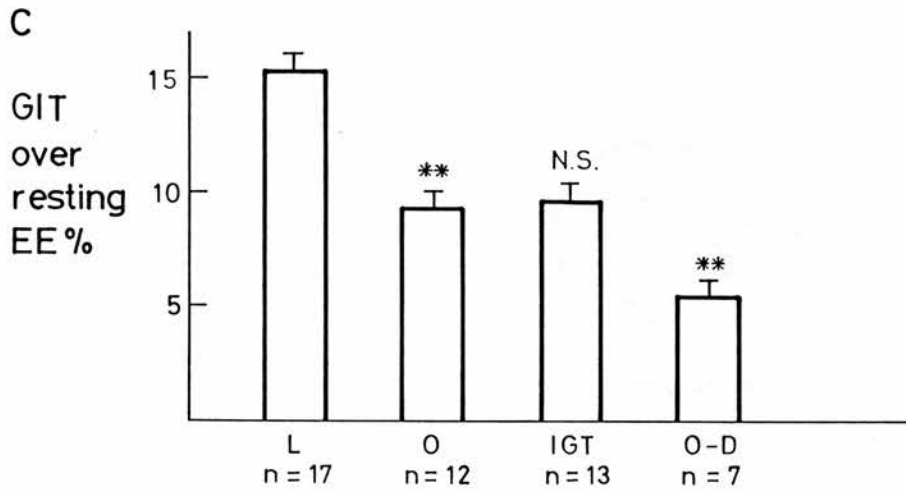
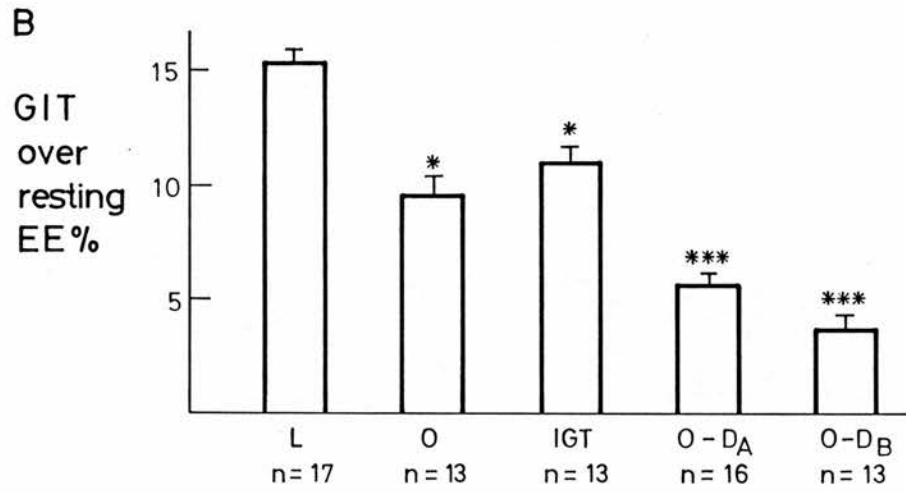
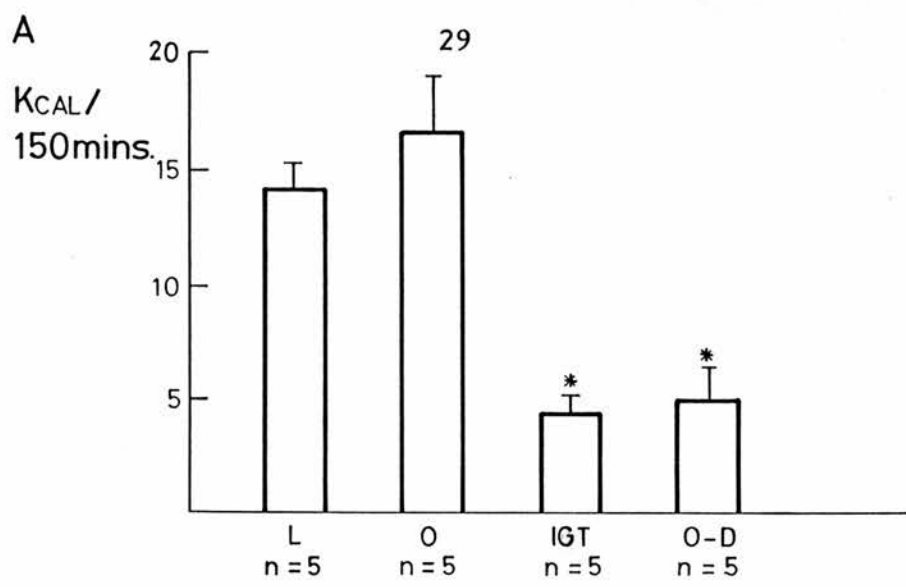
Interestingly, Allen and Dubois also studied the specific dynamic action of meals consisting of "97 g olive oil" or "125 mg oatmeal and 15 g butter" and observed no difference "as respects promptness and intensity" between diabetic and non diabetic subjects. They were obviously aware, although did not quote, Neumann's (1902) recent work and they comment "there was no indication of an abnormally high metabolism (Luxuskonsumption) on the excessive diet". It was not until almost 70 years later that Nair et al (1984) once again examined the resting metabolic rate in ketotic type I diabetic subjects and found it to be significantly elevated. Studies of non-shivering and diet-induced thermogenesis in Type I diabetes mellitus have however not been reported in the literature since the pre-insulin era.



The association of Type II diabetes with obesity has resulted in recent attention to the study of the thermogenesis in these subjects. However these studies have almost wholly concentrated on the thermic effect of a glucose load.

In contrast to those reports in obese subjects (Table 1.1) the thermic response to oral (Golay et. al., 1982; Schutz et. al., 1984b; Nair et. al., 1986) and intravenous (Ravussin et. al., 1983) glucose has been consistently demonstrated to be suppressed in obese diabetic and glucose intolerant subjects compared to lean subjects (Fig. 1.4). The study of Nair et. al. (1986) is interesting in two respects. Firstly it reports an elevation of the resting metabolic rate above predicted values not only in diabetic subjects but also those with impaired glucose tolerance. Secondly, the effect of glucose intolerance/diabetes on the blunting of the thermic response to glucose appeared to be independent to that of obesity in that the obese subjects in this study, with similar body weight had a normal thermic response to glucose. The data of Golay et. al. (1982) suggests that the degree of glucose intolerance may further impair the thermic response to oral glucose in that those subjects with a diminished insulin response to glucose (Fig. 1.4(b), O-D<sub>B</sub>) had the smallest thermic response. Thus, some of the thermic abnormalities noted in animal models and diabetes are paralleled in diabetic man but the picture is far from complete.

Fig. 1.4 (overleaf)



## 1.5. BROWN ADIPOSE TISSUE IN ADULT MAN

### 1.5.1. Anatomy

It is an interesting coincidence that in the same year that Neumann published his observations on "luxuskonsumption" an anatomist named Hatai (1902) made the observation of the similarity between the interscapular hibernating gland of certain rodents and the embryonal fat pad of human neonates. These findings remained confined to that of an esoteric piece of comparative anatomy until the demonstration of a function for this tissue in the human neonate as a site for non-shivering thermogenesis (Aherne and Hull, 1966). The first systematic study of the distribution of brown adipose tissue in the human at different age groups (Heaton, 1972) demonstrated a wide distribution of brown adipose tissue in all areas of the body in the first decade of life but this tended to diminish with increasing age with loss, in particular, from peripherally situated sites such as the interscapular area. However, the deeper sites such as those in association with the great vessels (cervical, pericardial and perirenal) tended to retain their brown fat up to the eighth decade.

Table 1.2 summarises all studies to date regarding the distribution of brown adipose tissue in adult man. All studies used post mortem material with the exception of Astrup et. al. (1984) who identified thermographically interscapular areas of heat production in response to ephedrine and obtained tissue at the maximal "hot spot" using a fine needle biopsy technique. The pooled data from this table illustrates the perirenal site as the major site of persistence of brown adipose tissue in adult man.

The positive identification of brown adipose tissue was based histologically on multilocularity of the adipocytes except in the study of Lean et. al. (1986) where a specific radio-immunoassay for uncoupling

No. of BAT positive samples (observed/total)

Study	Method	Age	Interscapular	Cervical	Perirenal
Heaton JM (1972)	Post mortem histology	20-80	4/31	24/31	24/31
Hassi J (1979)	Post mortem histology	14-80	1/26	13/32	12/34
Astrup A et. al. (1984)	Biopsy/histology	19-42	0/28	-	-
Astrup A et. al. (1985b)	Post mortem histology	20-50	-	4/10	141/174
Tanuma et. al. (1976)	Post mortem histology	20-49	-	-	68/85
Lean et. al. (1986)	Post mortem *RIA for uncoupling protein	19-96	-	-	5/17
		Total %	5/85 6%	41/73 56%	250/341 73%

Table 1.2: Histological occurrence of brown adipose tissue in man.

protein was used. It is interesting to note that in this study the highest proportion of positive samples was found in those from the axillary site (64%) which may represent a caudal extension of the cervical brown adipose tissue depot. When all these results are pooled the perirenal fat depot appears to be the most abundant source of brown adipose tissue in man and thus was chosen for the present study.

### **1.5.2. Evidence for Activation of Brown Adipose Tissue in Man**

#### **1.5.2.1. Phaeochromocytoma**

Several case studies report the presence of highly vascular brown fat tumours or "hibernomas" in association with catecholamine secreting phaeochromocytoma tumours (Rona, 1964; Leiphart and Nudelman, 1970; English et. al., 1973). The presence of uncoupling protein has also been demonstrated in perirenal fat obtained from patients with phaeochromocytoma (Ricquier et. al., 1982; Bouillard et. al., 1983) and may be elevated (Lean et. al., 1986) suggesting that activation of this tissue may occur in the presence of high concentrations of circulating noradrenaline.

#### **1.5.2.2. Thermography**

As an addendum to their classic demonstration of diet induced thermogenesis in cafeteria fed rats Rothwell and Stock (1979) showed thermographically increased blood flow to the interscapular area in one subject following the administration of an unspecified dose of ephedrine. This was interpreted as evidence of interscapular brown adipose tissue thermogenesis in man. These findings of increased blood flow in the interscapular area following ephedrine administration have been confirmed by other workers (Lev-Bari 1982; Astrup et. al., 1984) but the latter group

were unable to demonstrate the histological presence of brown adipose tissue in these areas (Table 1.2).

#### **1.5.2.3. Cold adaptation**

Huttunen et. al., (1981) examined the incidence and enzymic activity of brown adipose tissue in eleven outdoor and ten indoor workers at up to five days post mortem and concluded from these measurements of mitochondrial enzymes performed under variable conditions that there was evidence of increased brown adipose tissue activity in subjects with increased cold exposure. As the major adaptive response to cold in Western man (especially in Finland) is to diminish heat loss by adding another layer of insulation (i.e. clothing) it is unlikely that significant cold adaptation was observed in these subjects.

#### **1.5.3. Function of brown adipose tissue in man**

To date, although there is ample evidence of the histological presence of small amounts of brown adipose tissue persisting in adult man there is no evidence to support a functional role for this tissue in adaptive thermogenesis. The association of similar thermic abnormalities in animal models of obesity and human obesity, the demonstration of a possible adaptive thermogenic mechanism similar to the cafeteria feeding model in rats and finally the anatomical demonstration of brown adipose tissue in man has led to the speculation that this tissue may be responsible for adaptive thermogenesis in man and, further, that abnormalities of this tissue may be involved in the aetiology of obesity.

However, histological evidence of this tissue in adult man does not necessarily infer function. The inaccessibility of this tissue in man has rendered it particularly unamenable to study in vivo and therefore

experiments were designed (Chapter V) to attempt to examine the functional capacity of perirenal brown adipose tissue in man using indirect methods.

## 1.6. CONCLUSIONS

From the foregoing discussion it is apparent that several questions remain unanswered regarding the control of energy expenditure in adult man and thus the present studies were designed to attempt to clarify several points.

Firstly, it is not clear from the literature what the effect of abnormal glucose tolerance per se is on energy expenditure and thermogenesis in man in that studies examining this problem have almost exclusively been performed in subjects who are also obese. Therefore, I have measured several components of energy expenditure in non-obese type I diabetic subjects during a period of poor glycaemic control on conventional insulin therapy (Chapter III).

Secondly, to demonstrate if abnormalities of energy expenditure in these subjects are due to poor glycaemic control alone or to other metabolic abnormalities associated with diabetes mellitus, the type I subjects have been studied after a period of continuous subcutaneous insulin infusion to observe whether improvements in glucose tolerance results in a reversal of the thermic abnormalities (Chapter III).

Thirdly, in an attempt to identify whether thermogenic mechanisms have a therapeutic role in the management of type II diabetes mellitus, energy expenditure has been examined during biguanide therapy, which is documented to promote weight loss, and during sulphonylurea therapy, which tends to result in weight gain, in well controlled type II diabetic subjects (Chapter IV).



Finally, as the control of energy expenditure is firmly focussed on brown adipose tissue in animals, yet evidence of its functional capacity in man is lacking, I have attempted to assess the contribution of this tissue to noradrenaline induced thermogenesis in adult man (Chapter V).

## Chapter II

### Methodology

## 2.1. MEASUREMENT OF ENERGY EXPENDITURE IN MAN

### 2.1.1. Introduction

Calorimetry is the method of choice for studying energy expenditure in man (Garrow, 1978). Several calorimetric methods are available to the investigator based on the principles of either direct calorimetry which measures heat dissipated from the body or indirect calorimetry which measures heat production resulting from oxidative processes.

For the present study the requirements were that of a system capable of measuring resting metabolic rate (RMR) and the short term changes (less than three hours) resulting from oral and intravenous stimuli. The method should also be sensitive to artefact, have a fast reaction time and be comfortable and reproducible. The direct and whole body indirect calorimeters using a respiration chamber are unsuitable for experiments of less than five hours duration (Jequier, 1985) due to the slow equilibration time involved. Indirect methods using a Douglas bag technique and mouthpieces involve a certain amount of discomfort to the subject if the duration of the experiment exceeds 20 minutes resulting in an artefactual rise in metabolic rate (Garrow, 1978). A ventilated hood system was thus the method of choice and had the additional advantages of being relatively inexpensive and easy to maintain.

### 2.1.2. Indirect Ventilated Hood Calorimetry

RMR and the thermic responses to a test meal and intravenous infusion of noradrenaline were measured using an indirect computerised ventilated hood calorimeter based on an original system developed at the Medical Research Council Dunn Nutrition Laboratory, Cambridge.

Calibration of the oxygen and carbon dioxide meters was performed at the beginning and end of each experiment with oxygen free nitrogen (British Oxygen Corporation Ltd.), 0.8% carbon dioxide in air (British Oxygen Corporation Ltd.) and room air. To ensure constancy of the room environment during the experiments the calorimeter suite had a double door entry system.

All measurements were performed under thermoneutral conditions with subjects wearing light clothing and following a 10 hour overnight fast and abstinence from caffeinated beverages and cigarette smoking. Thermoneutrality is defined as that environmental temperature at which the body temperature is maintained whilst heat production and evaporative water loss are at a minimum (Hey, 1975). For the adult subject thermoneutrality was taken as 26-28°C (Itoh, 1974).

### 2.1.3. Resting Metabolic Rate

Basal metabolic rate is defined as the energy output of an individual under standardised conditions: bodily and mentally at rest 12-18 hours after a meal in a thermoneutral environment (Garrow, 1978). As it is difficult in practice to reproducibly achieve a total basal mental and physical state the term RESTING METABOLIC RATE (RMR) is used throughout as it is a more exact expression of that which is measured.

Measurements of RMR were performed between 0830 and 0930 hours with subjects lying semi-recumbent following 30-45 minutes equilibration at rest and after venous cannulation. Results are expressed as the mean  $\pm$  SEM of a 20 minute measurement.

The principle of indirect calorimetry arose from the meticulous direct calorimetric studies of Atwater and Benedict (1905), in which they observed that heat production could be accurately predicted from measurements of oxygen consumption and carbon dioxide production. Heat production was calculated from the product of oxygen consumption (l/min) and the calorific value of the oxygen determined from the respiratory quotient (RQ).

The ventilated hood system is illustrated in Figure 2.1. The semi-recumbent subject rested on the bed with their head enclosed in a loose fitting transparent hood through which room air was drawn at a constant rate of 45 l/min. Expired air was withdrawn continuously for measurement of oxygen (l/min) by paramagnetic analysis (Taylor, Servomex) and carbon dioxide (l/min) by infra-red analysis (SS-200, Analytic Development Company). Readings were taken at 1 minute intervals and the oxygen consumption and carbon dioxide production were calculated as illustrated in Table 2.1.

The standard formula of Weir (1949), based on a correction for the non-protein respiratory quotient, was used to calculate heat production.

$$\text{HEAT PRODUCTION (KJ/min)} = 16.494 \times \text{O}_2 \text{ consumption (l/min)} + 4.62 \times \text{CO}_2 \text{ production (l/min)}$$

Calculations were performed using a computer programme (Appendix 1) Copyright Dunn Nutrition Laboratory, Cambridge) which allowed immediate display of the parameters of O<sub>2</sub> consumption (l/min), CO<sub>2</sub> production (l/min), RQ and heat production (KJ/min) on a visual display unit. This facilitated the exclusion of artefactual results due to coughing, hyperventilation or movement.



Fig. 2.1 Ventilated hood calorimeter. Subject lying semirecumbent with P.V.C. hood in position. Expired air is drawn from the hood to the calorimeter which measures the concentration of expired oxygen and carbon dioxide. Calculation of heat production is facilitated by Commodore 8250 computer and results are immediately displayed on television screen.

$RO_2$  = Room oxygen concentration %

$RCO_2$  = Room carbon dioxide concentration %

$RN_2$  = Room nitrogen concentration %  $(100-RCO_2-RO_2)$

$EO_2$  = Expired oxygen concentration %

$ECO_2$  = Expired carbon dioxide concentration %

$EN_2$  = Expired nitrogen concentration %  $(100-ECO_2-EO_2)$

$V_{STPD}$  = Volume of expired air per minute at STPD

$$\text{True Oxygen} = \frac{EN_2}{RN_2} \times RO_2 - EO_2$$

$$\frac{\text{True Carbon Dioxide}}{(\text{CO}_2 \text{ production})} = ECO_2 - RCO_2$$

$$\text{Respiratory Quotient} = \frac{\text{True CO}_2}{\text{True O}_2}$$

$$\frac{\text{Oxygen Uptake}}{(\text{O}_2 \text{ consumption})} = V_{STPD} \times \frac{\text{True O}_2}{100} \quad \text{l/min}$$

Table 2.1 - Calculation of True Oxygen, True Carbon Dioxide, Respiratory Quotient and Oxygen Uptake.

#### 2.1.4. Ideal Body Weight

Ideal body weight (IBW) was defined as that given as an acceptable average weight in the report of the Royal College of Physicians (1983) based on the weight for height tables of the Metropolitan Life Insurance Company (1960).

#### 2.1.5. Thermic Responses

The thermic response to a meal or noradrenaline infusion is defined as the rise in heat production (KJ/min) above the resting value (RMR). Results are expressed as the mean integrated rise during the period of study (KJ) calculated from the area under the curve  $\pm$  SEM.

##### 2.1.5.1. Thermic response to a meal

A standard mixed liquid meal was administered following measurement of the RMR and consisted of one sachet Carnation Build Up (Vanilla Flavour), reconstituted in whole milk to give 32.6 KJ (7.8 Kcals)/kg ideal body weight. As subjects had the thermic responses to a meal measured on several occasions the caloric load was calculated on an IBW basis rather than total body weight to ensure a similar stimulus was given on each occasion. The meal was designed to be similar in energy content and constituents to that which diabetics would normally ingest each morning and had a protein:fat:carbohydrate ratio of 1:1.2:2.3 and contained approximately 50 g of carbohydrate. As most subjects will normally only tolerate about three hours under the ventilated hood without discomfort or a desire to micturate a liquid meal was chosen to facilitate the measurement of the thermic response within that time. Metabolic rate was measured for two hours continuously following replacement of the hood after meal ingestion and measurement of RMR.



Venous blood samples were obtained at -5, 0, 30, 60, 90 and 120 minutes following meal ingestion for analysis of plasma glucose.

#### 2.1.5.2. Thermic response to noradrenaline infusion

An intravenous 21 gauge cannula (Abbot) was placed in the contralateral brachial vein used for venous sampling. 0.9% normal saline (Boots p.l.c.) was infused at the same rate of noradrenaline infusion during the equilibration period and measurement of RMR.

Noradrenaline (Levophed, Winthrop), diluted in 0.9% saline was infused at the rate of  $0.1 \mu\text{g/kg IBW/minute}$  for 45 minutes and the metabolic rate was measured continuously. The dose infused was chosen to result in a plasma noradrenaline concentration corresponding to that found in moderate exercise and previous work using a similar protocol achieved peak plasma noradrenaline concentrations of  $1 \mu\text{g/L}$  (Jung, 1979). As with the meal the dose was calculated per IBW to ensure reproducibility of experimental conditions.

Venous blood for analysis of glucose, free fatty acid and glycerol concentrations were withdrawn at -5, 0, +15, +30 and +45 minutes during noradrenaline infusion.

#### 2.1.6. Predicted Metabolic Rate

Predicted metabolic rate (KJ/min) of each subject was calculated from the DuBois normal standards modified by Boothby and Sandiford (1929) based on the age, sex and surface area of the subject.

## 2.2. BLOOD ASSAYS

### 2.2.1. Venous Blood Sampling and Collection

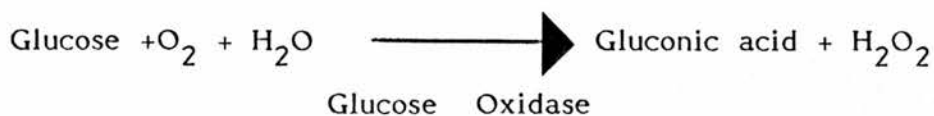
Venous blood samples were obtained using an indwelling 19 gauge cannula (Abbot) placed in a brachial vein. A three-way tap was used to lock the system; the cannula being kept patent with 0.5 ml 3.8% sodium citrate (British Pharmacopocia Codex) diluted 1 in 10 with 0.9% saline. Before any blood samples were taken the cannula was emptied of sodium citrate by withdrawing and discarding 2 ml of blood and citrate. Blood samples were collected as follows:

1. Into EDTA tubes (Searle) for insulin and HbA<sub>1</sub> estimation.
2. Into fluorate oxalate tubes (Searle) for plasma glucose estimation.
3. Into glass tubes (Searle) for serum collection for glycerol, free fatty acids and thyroxine, T3 and TSH estimations.

All tubes for plasma samples were cooled on ice, centrifuged at 2400 R.P.M. for 10 minutes and the plasma separated and frozen at -20°C until assayed.

### 2.2.2. Plasma Glucose

Plasma glucose was assayed using an enzymatic colorimetric method adapted from Trinder (1969) using a Technicon AAII glucose autoanalyser system. In this method glucose and oxygen are converted to gluconic acid and hydrogen peroxide by the enzyme glucose oxidase.



The chromogen used was 4-aminophenazone which when added to hydrogen peroxide in the presence of peroxidase results in the coloured product 4-(p-benzoquinone-mono-imino)phenazone.



Absorbance of this compound was measured on a spectrophotometer at 520 nm. Standard curves were constructed for concentrations of glucose of 1, 2.5, 5, 7.5, 10, 15, 20 and 25 mmol/l at the start and finish of each run. A drift standard of 7.5 mmol/l was included after every tenth unknown sample. The inter-assay coefficient of variation (C.V.) was 3.2% and intra-assay C.V., 2.0%.

### 2.2.3. Free Fatty Acids

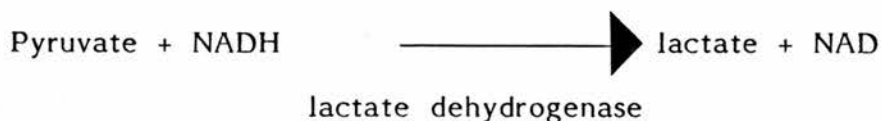
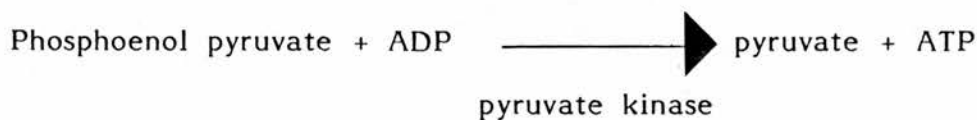
Serum free-fatty acids (FFA) were measured using an extraction photometric method adapted from Chromy *et al.* (1977). In this method 100  $\mu\text{l}$  of serum is mixed with a stable aqueous copper reagent and a chloroform-heptane-methanol (C-H-M) mixture. The copper ions in solution react quantitatively with the FFA to form a complex (FFA-Cu soap) which is extracted into the C-H-M upper phase.

Unreacted copper ions remain in the aqueous phase and an aliquot of the C-H-M phase is mixed with a chromogenic reagent (1-(2-thiazolylazo)-2-naphthol (TAN)). The absorbance of the coloured product was measured at 570 nm. Standards were prepared from a 2 mmol/l stock solution of sodium

palmitate in bovine albumin solution to give working solution concentrations of 0.1, 0.25, 0.50, 1.0, 1.25 and 1.50 mmol/l. The intra-assay C.V. was 8.4%.

#### 2.2.4. Glycerol

Serum glycerol was measured by a fully enzymatic method using a BCL triglyceride kit (No. 125032) with lipases and esterases removed. In this method glycerol is quantified by the following series of coupled enzymatic reactions.



The final reaction results in a decrease in absorbance at 340 nm as NAD is formed from NADH and is equivalent to the amount of glycerol originally present in the sample. A Rotachem II a centrifugal fast analyser was used for the assays using 50  $\mu\text{l}$  serum and 250  $\mu\text{l}$  reagent. The setting standard of 300  $\mu\text{mol/l}$  and high (80  $\mu\text{mol/l}$ ) and low (10  $\mu\text{mol/l}$ ) quality controls were prepared from Analar glycerol (BDH, 10118). The inter-assay C.V. was 3% and the intra-assay C.V. 2%.

### 2.2.5. Haemoglobin A<sub>1</sub>

Haemoglobin A<sub>1</sub> (HbA<sub>1</sub>) was measured in the routine Diagnostic Laboratory, Ninewells Hospital, using the Glytrac<sup>TM</sup> Corning apparatus. The principle of the test is based on the observation that the non enzymatic addition of a glucose molecule to the terminal valine residue of both beta chains of HbA results in a loss of net positive charge. Thus, under the influence of electroendosmotic flow HbA<sub>1</sub> migrates further from the origin than HbA on an electrophoretic gel. Densitometer scanning of the gel at 420 nm allow quantification of the amount of total HbA<sub>1</sub> present. Stable HbA<sub>1</sub> was measured after the removal of labile fraction (Labile Removing Haemolyzing Reagent, Corning Ltd.). The reference range for the non-diabetic adult population from this laboratory is 4.9 to 7.9%.

### 2.2.6. Thyroid Function

Thyroxine (T<sub>4</sub>), tri-iodothyronine (T<sub>3</sub>) and thyroid stimulating hormone (TSH) were measured in the routine diagnostic laboratory, Ninewells Hospital, using 'in-house' radioummunoassay systems. Thyroxine was measured using a single antibody technique with radiolabelled T<sub>4</sub> (SAPU; Scottish Antibody production unit) precipitated with polyethylene glycol (PEG). T<sub>3</sub> and TSH were measured using a double antibody assay (SAPU).

## 2.3. ETHICAL APPROVAL

Informed consent was obtained from all subjects and ethical approval for each study was given by Tayside Health Board Ethical Committee in accordance with the Declaration of Helsinki.

#### **2.4. STATISTICAL ANALYSIS**

Statistical analysis used standard parametric tests. The students t-test was used for unpaired observations and the paired t-test for paired observations. Regression analysis and correlation coefficient of observed and predicted values were calculated using 'Statspak' computer programme.

Chapter III

Energy Expenditure in Type I Diabetes Mellitus

## SUMMARY

The hypothesis that insulin has a role in the control of body weight via alterations in energy expenditure was tested.

Resting metabolic rate (RMR) and the thermic responses to a mixed meal and the infusion of noradrenaline were measured in nine lean Type I diabetic subjects initially while poorly controlled on conventional therapy (CT) and then when optimally controlled on continuous subcutaneous insulin infusion (CSII). The response to fat supplementation for one week was also assessed and the results compared to eight non-diabetic subjects who acted as controls.

1. CSII resulted in marked improvement of glycaemic control and a mean gain in weight of 3.2 kg.
2. While on CT the RMR in the diabetic subjects was elevated but returned to predicted values on CSII.
3. CSII corrected the reduced thermic response to the meal after one week of fat supplementation while controlled on CT.
4. The thermic response to infused noradrenaline was reduced by 50% in the diabetic subjects irrespective of the degree of glycaemic control.

These findings suggest that the reduction in energy expenditure contributes to the gain in weight observed on optimisation of diabetic control.

The wider implications of these findings in relation to an understanding of the pathophysiology of abnormalities of energy balance such as the obese state are discussed.



### 3.1. INTRODUCTION

In Chapter I three recent interrelated areas of research were reviewed: firstly, the well established association of obesity with insulin resistance in man and laboratory rodents; secondly, the observations in animal models of obesity (Rothwell and Stock, 1981; Cunningham et. al., 1983; Seydoux et. al., 1983) and obese man (Golay et. al., 1982; Ravussin et. al., 1983) suggesting a requirement for insulin in sympathetic and diet induced regulatory thermogenesis; and finally the evidence implicating abnormalities of thermogenesis in the aetiology of obesity (Jung et. al., 1979; Shetty et. al., 1981). From these three strands a model has been proposed in which insulin responsiveness may act as part of the mechanism of controlling energy balance in man. Furthermore, it has been postulated that abnormalities of insulin sensitivity may itself predispose to obesity (Felig, 1984).

However, one of the confounding factors in elucidation of the role of insulin in modulating energy expenditure from both the rodent models and the human subjects studied to date is the inextricable relationship of insulin resistance to the obese state per se.

The present study was therefore designed to examine resting energy expenditure and thermic responses to food and noradrenaline in a group of non-obese subjects with abnormal glucose tolerance.

Normal weight Type I diabetic subjects were studied and compared to lean non-diabetic controls.

The two initial questions posed in this study were:

- 1) Does the lean poorly controlled Type I diabetic demonstrate similar abnormalities of RMR, and the thermic responses to food and noradrenaline infusion, to the obese insulin resistant subject?



and, if so

- 2) Are these abnormalities reversible on attainment of optimal glycaemic control?

In order to assess any role that insulin might have in the adaptive thermic response to overfeeding, a further study was designed in which the thermic response was studied before and during a period of excess calorie ingestion.

An understanding of the hormonal signals that control energy balance may lead to a method of therapeutically manipulating this homeostatic mechanism in pathological states such as obesity.

### 3.2. EXPERIMENTAL PROCEDURE

The study design is illustrated in Figure 3.1. Eight normal weight, poorly controlled Type I diabetic subjects and eight lean non-diabetic subjects were studied. The RMR and the thermic response to a mixed meal and noradrenaline were measured before and during one week of fat supplementation of the diet in both groups of subjects.

The diabetic subjects were then commenced on continuous subcutaneous insulin infusion and the measurement of the RMR and thermic responses were repeated when optimal control had been achieved.

#### 3.2.1. SUBJECTS

All subjects had a stable body weight for at least six months prior to commencement of the study and none were obese as defined by a body

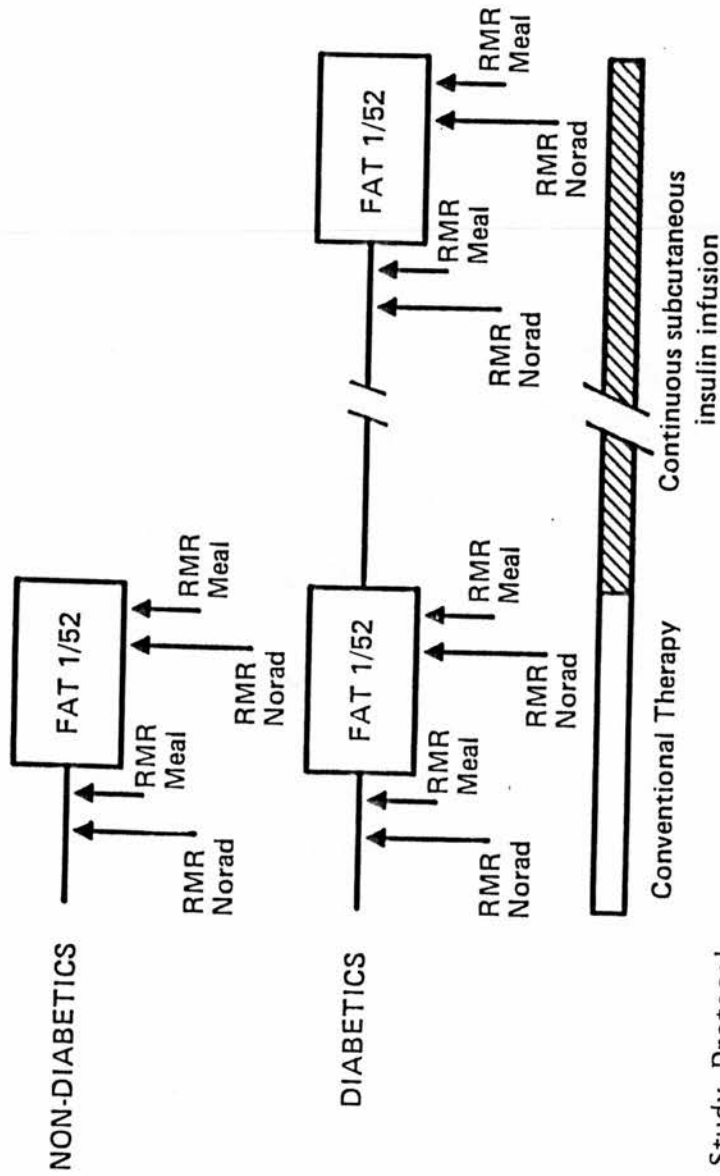


Fig. 3.1. Study Protocol

RMR and thermic response to noradrenaline (RMR+Norad) and RMR and thermic response to mixed meal (RMR+meal) were measured before and during one week's supplementation of the diet with fat (Section 3.2.4) in non diabetic and diabetic subjects. The responses in diabetic subjects were repeated following attainment of glycaemic control using continuous subcutaneous insulin infusion

mass index (BMI) (weight (kg)/height<sup>2</sup>(m)) of greater than 27. The Report of the Royal College of Physicians on Obesity (1983) based on the weight for height tables of the Metropolitan Life Insurance Tables (1960) defines obesity as a BMI of greater than 30 in men and greater than 28.6 in women.

Two diabetic subjects were cigarette smokers (<10/day) and one non-diabetic smoked cigarettes (15/day). None of the female subjects had been breast-feeding for at least two years prior to the study. None of the subjects were receiving regular oral medication and all were clinically and biochemically euthyroid (Table 3.1).

#### 3.2.1.1. Diabetic Subjects (Subjects 1-9)

Nine diabetic subjects controlled on a twice daily short and intermediate acting insulin regimen were recruited from Ninewells Hospital Diabetic Clinic during the period January to August 1984. Routine screening for complications of diabetes mellitus was performed in all subjects. All subjects had normal erect and supine blood pressure measurements, clinical motor and sensory nerve function, plasma urea and creatinine concentrations and were negative for urinary protein on Labstix (Ames Ltd.) testing. Subject No. 8 had background diabetic retinopathy; the remaining subjects had no evidence of diabetic changes on retinal examination. Subject details are illustrated in Table 3.2.

Subjects were selected on the basis of poor control ( $HbA_1 > 10\%$ ) despite good knowledge of diabetes and were enthusiastic about improving control on continuous subcutaneous insulin infusion.

	Age (yrs)	Sex M:F	Weight (kg)	BMI (kg/m <sup>2</sup> )	T <sub>4</sub> (nmol/l)	T <sub>3</sub> (nmol/l)
Controls (n = 8)	26.3 ± 3.4	2:6	59.8 ± 4.8	21.7 ± 1.7	95 ± 16	1.70 ± 0.15
Diabetic Subjects (n = 8)	31.2 ± 8.1	2:6	67.9 ± 12.0	23.8 ± 2.7	99 ± 14	1.65 ± 0.26

Table 3.1: Characteristics of controls (n = 8) and diabetic subjects (n = 8).

Figures expressed as mean ± SD.

Diabetic subject No. 8 was excluded from this analysis as he did not complete the protocol (Section 3.3.1.1). There were no statistically significant differences between controls and diabetic subjects.

Subject	Age	Sex	Weight (kg)	BMI (kg/m <sup>2</sup> )	HbA <sub>1c</sub> on CT (%)	Duration of Diabetes (yrs)	C-PEPTIDE Fasting (see Section 3.3.2) (nmol/l)	Stimulated
1	34	M	88.0	25.9	12.1	0.4	0.14	0.22
2	36	F	48.7	18.1	10.3	3.5	0.03	0.03
3	37	F	66.7	24.5	10.5	3	<0.01	<0.01
4	44	M	79.6	25.4	12.4	3.5	0.04	0.10
5	34	F	63.9	25.6	12.1	4.5	<0.01	<0.01
6	26	F	64.5	23.2	10	4.5	0.03	0.01
7	21	F	60.4	21.7	15.6	3	0.04	0.07
8	30	M	79.8	27.3	11.1	16	0.04	0.06
9	18	F	71.6	26	14.4	12	<0.01	<0.01
Mean	31.1	6F:3M	69.2	24.2	12.0	5.9	-	-
± SD	8.2		±1.9	±2.8	±2.0	±4.9		

Table 3.2: Characteristics of diabetic subjects

### 3.2.1.2. Non-diabetic Subjects

Eight non-diabetic subjects with no family history of obesity or diabetes were recruited from hospital staff and the community and are hereafter referred to as 'controls'. Subject characteristics are shown in Table 3.1.

### 3.2.2. C-PEPTIDE MEASUREMENT

To confirm the Type I nature of the diabetic subjects C-peptide was kindly measured by Dr M. Small, Glasgow Royal Infirmary. (RIA kit for human C-peptide; Novo Research Institute). Plasma samples were obtained fasting and also 30 minutes following meal ingestion and frozen and stored at  $-20^{\circ}\text{C}$ . The results are shown in Table 3.2. The normal fasting reference range for this assay is 0.18 to 0.63 nmol/l. Subject No. 1 had been diagnosed diabetic five months prior to the study and his results indicate a detectable but clinically insignificant level of endogenous insulin reserve. As he had presented acutely in diabetic ketoacidosis he can be considered a Type I diabetic.

### 3.2.3. CONTINUOUS SUBCUTANEOUS INSULIN INFUSION

#### 3.2.3.1. General Principles

Continuous subcutaneous insulin infusion (CSII) was initially developed in the mid-1970's as a method of insulin delivery with the potential capability of achieving long-term euglycaemia in order to examine further the role of glycaemic control in the development of diabetic complications (Pickup et. al., 1978).

Most recently the application of CSII has extended from its limited use as a research tool in a few specialist centres to that of a practical alternative method of insulin delivery in selected diabetic patients (Ward, 1984).

At the time of study design there was considerable enthusiasm based on the demonstration of marked improvements in glycaemic control and achievement of normal or near normal HbA<sub>1c</sub> concentrations on CSII compared to conventional therapy (Calabrese et. al., 1982; Home et. al., 1982). However since then several reports have highlighted the disadvantages of an insulin regimen based solely on short-acting insulin (Anonymous, 1985).

Preliminary short term studies also suggested that the recorded insulin resistant state observed in Type I diabetic subjects (Del Prato et. al., 1983) was reversible on optimal glycaemic control using CSII (Lager et. al., 1983; Yki-Jarvinen and Koivisto, 1984). Thus CSII was employed as the method of improving glycaemic control in our subjects.

#### 3.2.3.2. Practical Aspects

On recruitment to the study the potential benefits and hazards of CSII were discussed with each patient and the need for frequent home glucose monitoring was emphasised.

Subjects were then admitted to the ward for a period of 2 to 3 days to commence CSII (Travenol AS6C-UI100 pump; Travenol Ltd.) of Actrapid MC (Novo) insulin and familiarise themselves with the procedures involved. During this time they were instructed on the adjustment of the single basal infusion rate to achieve a fasting and early morning nadir (3 a.m.) blood glucose of between 4 and 6 mmol/l using BM Stix (Boehringer-Mannheim Ltd.) in a reflectance meter (Reflolux, Boehringer-Mannheim Ltd.) Pre-prandial bolus doses were adjusted to achieve a 2 hour post-prandial rise of 2 mmol/l. During illness subjects were advised to increase the basal rate of infusion and if necessary to give more frequent boluses. Patients were instructed to change the infusion site daily, the cannula every 2 days



and the syringe weekly.

All patients were instructed to keep a supply of conventional syringes and insulin available in case of pump failure.

Frequent telephone contact was established during the first few weeks of therapy and thereafter the patients had open access to telephone advice from me at all times.

Once established on CSII, patients were advised to perform at least two blood glucose measurements daily and were reviewed at the Clinic at two-monthly intervals when HbA<sub>1c</sub> was monitored.

#### 3.2.4. Overfeeding

Fat is one of the major components of the Western diet resulting in excess energy intake and requires minimal expenditure for storage as adipose tissue (Flatt, 1978).

As facilities were not available for formal supervised overfeeding of the subjects in a metabolic ward the effect of supplementing their normal diet with 284 ml (1/2 pint) fresh double cream per day for seven days on the RMR and thermic responses was examined. This is equivalent to an energy rise of 5.23 MJ/day (1270 Kcal/day) and has fat:protein:carbohydrate ratio of 24:0.7:1.0. As this "overfeeding" was unsupervised and the precise calorie intake could not be documented, it is referred to hereafter as "fat supplementation".

The thermic responses to the meal and noradrenaline were measured on the sixth and seventh days of fat supplementation in random order. The RMR was measured on both mornings and expressed as the mean of these two measurements.

### 3.2.5. Insulin Regimens for Diabetic Subjects on Study Days

#### 3.2.5.1. RMR and Noradrenaline Infusion

- On Conventional Therapy

Subjects on conventional therapy were instructed to inject their normal dose of short acting insulin before the evening meal of the day prior to study. The injection of intermediate acting insulin was delayed until 2100 hours to prevent fasting hypoglycaemia the following morning during measurements.

RMR and the thermic response to noradrenaline were measured prior to the morning injection of short and intermediate acting insulins which were given at the end of the tests 20 minutes prior to breakfast.

- On CSII

Subjects continued the basal rate of infusion throughout the measurements and the morning pre-prandial bolus of short acting insulin was given prior to breakfast at the termination of measurements.

#### 3.2.5.2. Meal

- On Conventional Therapy

Subjects injected their normal doses of short and intermediate acting insulins following the measurement of RMR and 20 minutes prior to ingestion of the meal.

- On CSII

The morning pre-prandial bolus of short acting insulin was delivered following RMR measurement and 20 minutes prior to meal ingestion.

### 3.2.6. Insulin Withdrawal

In order to determine that the effects of CSII on resting metabolic rate were the result of insulin delivery and not merely one to 'training' of the subjects to the procedures involved, the RMR was measured on one further occasion 11 hours following the withdrawal of insulin.

Subjects were instructed to remove the infusion pump and cannula at 10 p.m. the evening prior to having their RMR measured at 9 a.m. The subjects were then recommenced on CSII and normoglycaemia restored within 4-5 hours. The RMR on conventional therapy and CSII was then compared to that following insulin withdrawal.

## 3.3. RESULTS

### 3.3.1. Continuous Subcutaneous Insulin Infusion

The results of CSII are presented in two sections.

Firstly the experience, based on the nine patients studied, with this method of insulin delivery will be reported with reference to patient acceptability, glycaemic control and complications of CSII during the first 124 patient months.

Secondly, the levels of glycaemic control that were observed at the time the subjects' RMR and thermic responses were repeated on CSII are reported.

### 3.3.1.1. General CSII Experience

#### Patient Acceptability

Eight of the nine diabetic subjects found CSII a highly acceptable method of insulin delivery which not only resulted in significant objective improvement of glycaemic control but also subjective improvement in lifestyle with increased flexibility of mealtimes, leisure and work activities.

Complications - Local cutaneous reactions. One subject (No. 8), who worked up to 18 hours per day as a market gardener, suffered persistent discomfort at the cannula insertion site and despite the use of alternative insertion sites (loin, thigh) was withdrawn from CSII after 10 months when a subcutaneous abscess developed at a needle insertion site. As this subject failed to achieve a  $HbA_{1c}$  of below 10% the metabolic studies on CSII were not performed and he was excluded from further analysis.

Five months following completion of the study, subject No. 6 developed abdominal lipohypertrophy which resolved on increased rotation of injection sites.

#### Hyperglycaemia

To date (June 1986) none of the subjects have had an episode of frank ketoacidosis but based on subjects' own records of home blood glucose monitoring acute minor episodes of loss of glycaemic control with blood glucose concentrations of up to 18 mmol/l were relatively common. Home blood glucose estimations of greater than 14 mmol/l were recorded at a frequency of one episode per 2.8 patient months during the first 124 patient months of CSII.

The vast majority of these episodes were associated with failure of insulin delivery as a result of blocked cannulae, air in the cannulae or luer lock leakage but a significant proportion were due to "patient malfunction" with failure to change batteries, fill the syringe or omission of pre-meal boluses. It is a credit to the patients' diligent home glucose monitoring that all these episodes were aborted within 12 hours.

### Hypoglycaemia

This can be considered under two headings - mild symptomatic (adrenergic symptoms only) and severe hypoglycaemia.

Mild symptomatic hypoglycaemia - Data collected retrospectively from patients' home glucose monitoring and diary recording of mild hypoglycaemia tends to be, by definition, selective and therefore these figures have not been formally quantified in terms of episodes per patient month.

However, subjectively the subjects noted an increased incidence of mild hypoglycaemia on CSII compared to conventional therapy, especially in the early months of treatment due to miscalculation of the pre-prandial bolus in relation to the carbohydrate content of the meal and exercise.

Most patients noted a different character to the reactions; symptoms such as perioral paraesthesiae - which had not been previously noted by five of the nine subjects on conventional therapy - became a prominent premonitory feature. Symptoms such as this tended to be slower in onset and consequently mild reactions could be swiftly aborted with carbohydrate.

Severe hypoglycaemia - This is considered as a hypoglycaemic reaction outwith the control of the patient requiring intervention from either a relative and/or a medical attendant due to neuroglycopenic symptoms.

With this clearly defined end point, quantification of episodes can be attempted. In 124 patient months three of the nine subjects developed severe hypoglycaemic reactions requiring intramuscular glucagon and/or hypertonic intravenous glucose. This represents a rate of one episode per 31 patient months in this small sample. Two of the subjects (Nos. 5 and 6) had never previously experienced severe hypoglycaemia.

Each of these three episodes was precipitated by miscalculation of the pre-prandial bolus in relation to meal size and exercise and two were further exacerbated by alcohol.

Pregnancy - It is interesting to note that one of the subjects (No. 5) became pregnant after seven months on CSII and was maintained in CSII throughout the pregnancy with no episodes of metabolic decompensation. The HbA<sub>1c</sub> at estimated time of conception was 7.8% and the mean HbA<sub>1c</sub> during pregnancy (mean  $\pm$  SD of six estimations taken at 8, 14, 18, 22, 28 and 34 weeks) was  $7.6 \pm 0.4\%$ . The final trimester of pregnancy was complicated with polyhydramnios and foetal heart slowing and a baby girl was delivered at Caesarian section at 37 weeks gestation weighing 3.9 kg. This figure is above the 95th centile for later-born girls with adjustments for maternal weight and height according to the Tables for Tanner and Thomson (1970).

#### 3.3.1.2. Glycaemic control

The mean HbA<sub>1c</sub> was significantly lower on CSII compared to conventional therapy (CT) at 3, 6, 9 and 12 months following institution of CSII (Figure 3.2).

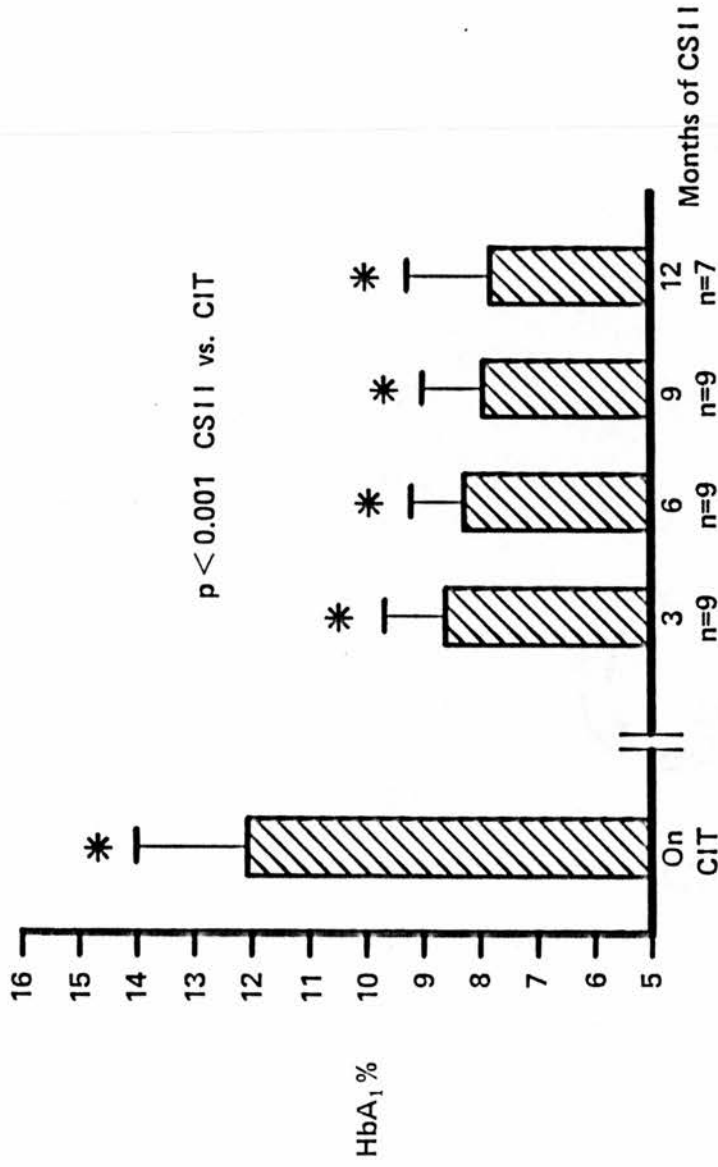


Fig. 3.2 Mean HbA<sub>1c</sub> % ( $\pm$  S.D.) on conventional insulin therapy (CIT) and during CSII therapy

The mean  $\text{HbA}_{1c}$  taken on the first morning of repeat metabolic studies (RMR and thermic responses) is shown in Table 3.3.  $\text{HbA}_{1c}$  had significantly decreased ( $p < .001$ ) despite similar total daily insulin dose and fasting blood glucose was similar to that in the non-diabetic control group (Table 3.4). The median time between study on CT and CSII was 5.3 months with a range of 3.5 to 8 months.

### 3.3.2. Weight

The weight of the eight diabetic subjects who completed the study rose significantly on CSII. These results are shown in Table 3.3 and refer to the body weight measured on the first morning of metabolic study on CT and CSII.

The mean change in body weight during the first nine months of CSII is shown in Figure 3.3. Following completion of the metabolic studies further dietary advice was given to the subjects to reverse this trend and therefore only the data during the first nine months is shown in this Figure. Subject No. 5 was excluded from this analysis as she had become pregnant (Section 3.3.1.1).

### 3.3.3. Resting Metabolic Rate

#### 3.3.3.1. On normal diet

The poorly controlled diabetic subjects had an increased RMR compared with predicted values (Table 3.5). The estimated difference was 346 KJ (83 kcal) per 24 hours with 95% confidence limits of 18.7 and 672.5 KJ (4.5 and 160.7 kcal). The RMR decreased to within the predicted range with CSII despite a significant increase in weight. The decrease with precise glycaemic control reduced energy expenditure by an estimated 374.4 KJ (89.5 kcal) per 24 hours (95% confidence limits 93.6 and 655.2 KJ (22.4 and



	CT (n = 8)	CSII (n = 8)
HbA <sub>1c</sub> (%)	12.1 ± 2.0	7.5 ± 0.6 ***
Total Daily Insulin Dose (u/day)	42.8 ± 3.8	43.6 ± 4.5 (NS)
Weight (kg)	67.9 ± 12.0	71.4 ± 1.4*

\*\*\* p<0.001

\* p<0.05

NS Not significant

paired t-test

Table 3.3: HbA<sub>1c</sub> (%), total daily insulin dosage (u/day) and weight (kg) in the 8 diabetic subjects on conventional therapy (CT) and continuous subcutaneous insulin infusion (CSII). Figures expressed as mean ± SD.

<u>Subjects</u>	<u>Basal glucose mmol/l</u>		<u>Accumulative incremental glucose rise mmol/l.min</u>	
	<u>Usual Intake</u>	<u>Fat Supplemented</u>	<u>Usual Intake</u>	<u>Fat Supplemented</u>
Non diabetic	5.6 ± 0.24	5.5 ± 0.14	77.1 ± 30.1	57.32 ± 33.6
Diabetic on CT	11.6 ± 0.95	16.4 ± 1.30 <sup>††</sup>	427.3 ± 79.7 <sup>***</sup>	235.1 ± 95.4
Diabetic on CSII	7.1 ± 1.60 <sup>+++</sup>	4.5 ± 0.84 <sup>+++</sup>	387.0 ± 789.8 <sup>**</sup>	403.7 ± 59.6 <sup>**</sup>

Table 3.4: Plasma glucose responses to a meal before and during fat supplementation.

Mean ± SEM.

CT = Conventional therapy

CSII = Continuous subcutaneous insulin infusion

Diabetic on CT or CSII vs non diabetic \*\* p<0.02; \*\*\*p<0.01.

Diabetic on CT; fat supplemented vs usual intake <sup>††</sup>p<0.02.

Diabetic on CSII vs diabetic on CT <sup>+++</sup>p<0.001.

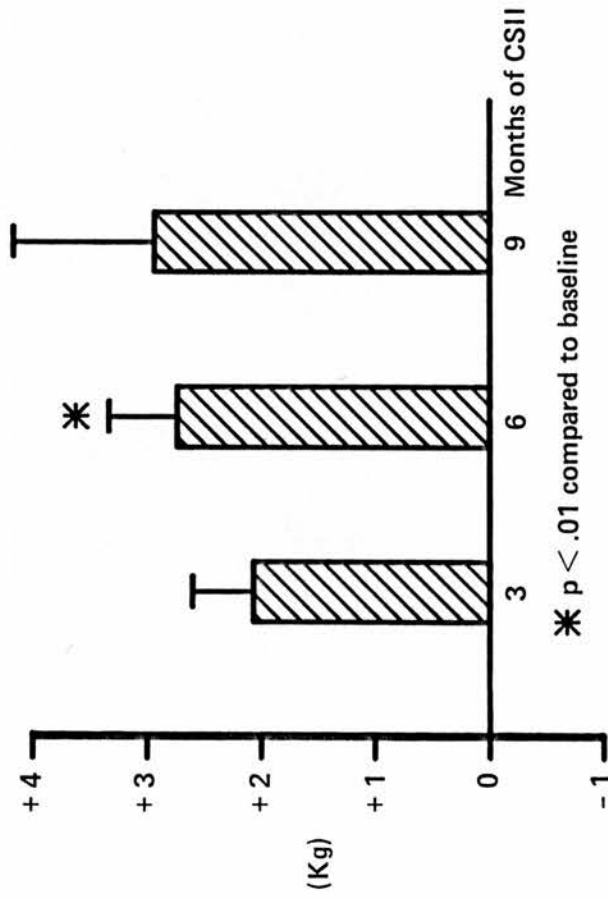


Fig. 3.3 Mean weight change (kg ± SEM) from baseline during CSII (n = 8)

<u>Subjects</u>	<u>Predicted</u>	<u>Observed</u>	
		<u>Usual Intake</u>	<u>Fat Supplemented</u>
Non diabetic	4.37 ± 0.13	4.24 ± 0.12	4.25 ± 0.19
Diabetic on CT	4.69 ± 0.26	4.93 ± 0.27 <sup>+</sup>	4.86 ± 0.24
Diabetic on CSII	4.69 ± 0.26	4.67 ± 0.34**	4.81 ± 0.37

Table 3.5: Predicted and observed value for the resting metabolic rate (kJ/min).

CT = Conventional therapy

CSII = Continuous subcutaneous insulin infusion

Diabetic on CT, observed on usual intake vs predicted; <sup>+</sup> p<0.05.

Diabetic on usual intake; CSII vs CT, \*\*p<0.02.

156.6 kcal)). The non-diabetic subjects showed a good correlation between observed and predicted values ( $r = 0.93$ ,  $p < 0.0005$ ).

#### 3.3.3.2. RMR on fat supplemented diet

There was no significant change in the RMR with fat supplementation in either the non-diabetic subjects or the diabetic subjects during conventional treatment or CSII (Table 3.5).

#### 3.3.3.3. Insulin withdrawal

Six subjects agreed to take part in the insulin withdrawal study (Nos. 1, 2, 3, 6, 7, and 9). Fasting plasma glucose rose over three-fold on insulin withdrawal and was associated with a 16% elevation of the RMR (Fig. 3.4).

With the small numbers involved there was no correlation ( $r = 0.17$ ) between the magnitude of the rise in plasma glucose and that of the RMR.

#### 3.3.4. Thermic Response to the Meal

##### 3.3.4.1. Normal diet

There was no significant difference in the energy content of the test meal between diabetic ( $1.80 \pm 0.13$  MJ) and control subjects ( $1.97 \pm 0.16$  MJ). Following ingestion of the meal, the control group and the diabetic subjects on CT and CSII had similar incremental thermic responses (Table 3.6 and Figure 3.5).

##### 3.3.4.2. Fat supplemented diet

When supplemented with fat the control group showed no change in the thermic response to the meal. In contrast, the diabetic subjects on conventional therapy exhibited a significant reduction of the thermic response during the second hour ( $p < 0.05$ ) which was 57% less than that

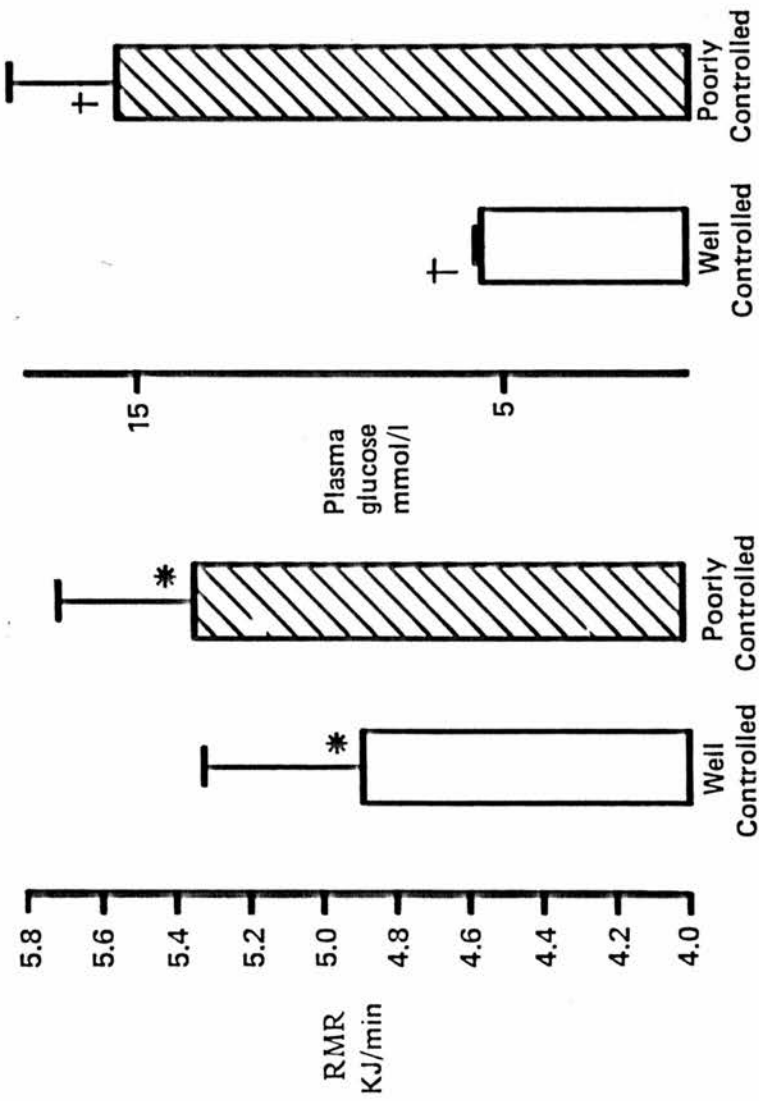


Fig. 3.4. Effect of insulin withdrawal on resting metabolic rate (RMR) and fasting plasma glucose in diabetic subjects ( $n = 6$ ) (Section 3.3.4.3)  
 $*p < 0.001$ ;  $+p < 0.01$ .

Subjects	TEST MEAL		NORADRENALINE INFUSION	
	Total (kJ)	1st hr (kJ)	2nd hr (kJ)	Total (kJ)
Non diabetic	Usual intake	72.2±8.4	34.6±3.4	37.5±5.3
	Fat supplement	78.0±9.4	37.6±6.4	40.4±11.2
Diabetic on CT	Usual intake	73.6±12.3	33.1±5.7	40.5±7.1 <sup>+</sup>
	Fat supplement	44.8±14.0	27.5±9.1	17.3±6.3 <sup>+</sup>
Diabetic on CSII	Usual intake	71.0±14.6	33.9±6.3	37.1±9.9
	Fat supplement	71.0±9.9	32.5±4.6	38.5±5.9
				13.4±3.7**
				16.1±1.9

Table 3.6 Accumulative incremental thermic response to the test meal and noradrenaline infusion before and during fat supplementation.

Mean ± SEM

CT = Conventional therapy.

CSII = Continuous subcutaneous insulin infusion.

Test meal responses compared in 8 diabetic and in 8 non diabetic subjects.

Noradrenergic response compared in 7 diabetic and in 8 non diabetic subjects.

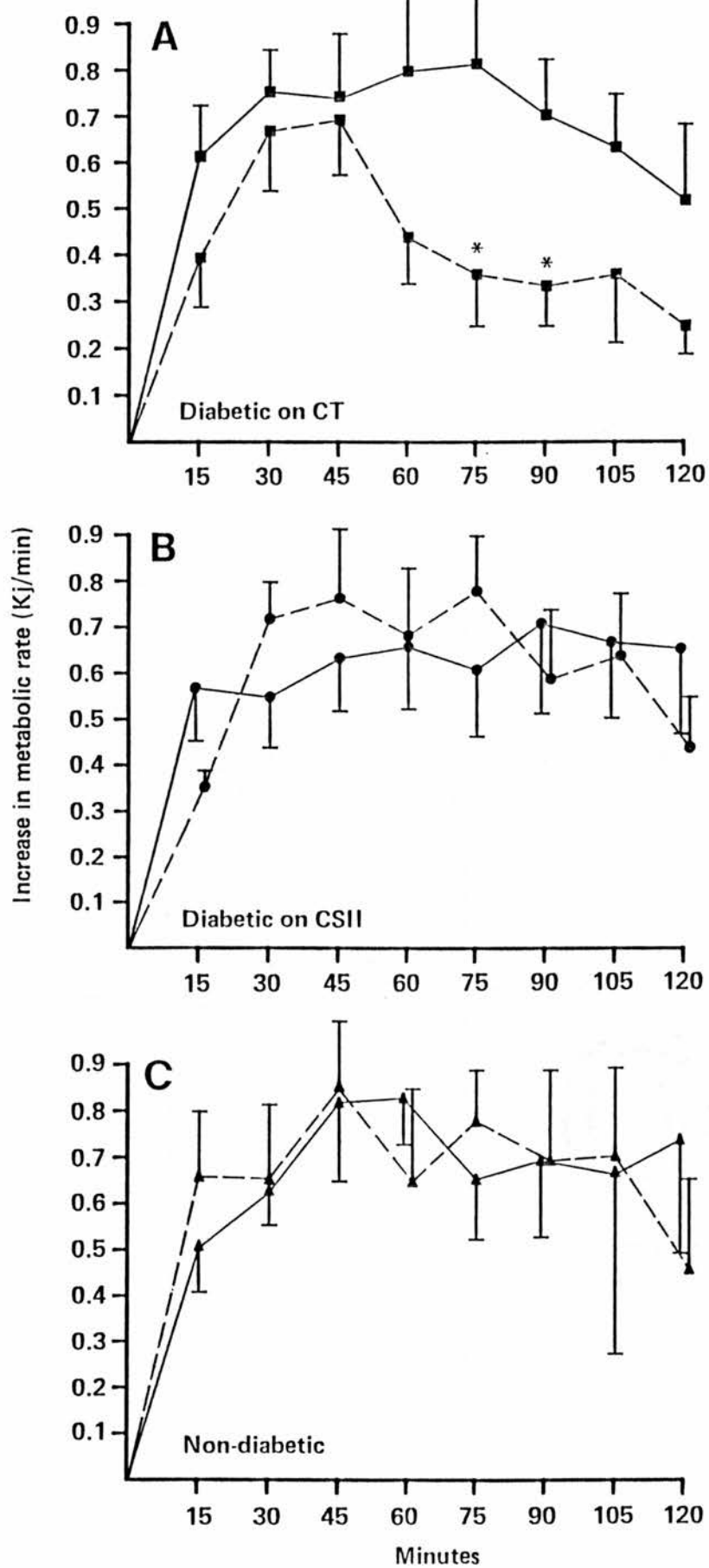
Diabetic on CT or CSII vs non diabetic (comparing usual diet vs usual diet; fat supplement vs fat supplement); \*p<0.05, \*\*p<0.02, \*\*\*p<0.01.

Diabetic on CT; fat supplement vs usual diet; <sup>+</sup>p<0.05.



Fig. 3.5 (overleaf)





observed in the same subjects before fat consumption. When these subjects had attained optimal glycaemic control on CSII, they then exhibited no significant change in their thermic response to the meal during fat supplementation.

### **3.3.5. Glucose Response to the Meal**

The fasting plasma glucose and following ingestion of the meal is shown in Figure 3.6. The mean plasma glucose was significantly lower at all times on CSII compared to CT and the mean fasting plasma glucose on CSII was not significantly different from the control subjects. The plasma glucose however following meal ingestion in the diabetic subjects on CSII still remained significantly higher than in the non-diabetic controls.

The accumulative incremental rise of glucose in response to the meal (Table 3.4) was 5.5-fold higher in the poorly controlled diabetic subjects compared to controls. On CSII this figure still remained five-fold greater than in the controls.

Fat supplementation resulted in a significant rise in fasting blood glucose in the diabetic subjects on CT but no change ( $p < 0.02$ ) during CSII or in control subjects (Table 3.4).

### **3.3.6. Thermic Response to Noradrenaline**

There was a significant increase in energy expenditure during noradrenaline infusion in all subjects.

Seven of the eight diabetic subjects demonstrated a blunted response to infused noradrenaline compared to controls (Fig. 3.7 and Table 3.6).

One diabetic subject (No. 6) was excluded from this analysis as the infusion of noradrenaline produced a subjective feeling of stress associated with restless legs and hyperventilation. This resulted in an exaggerated and

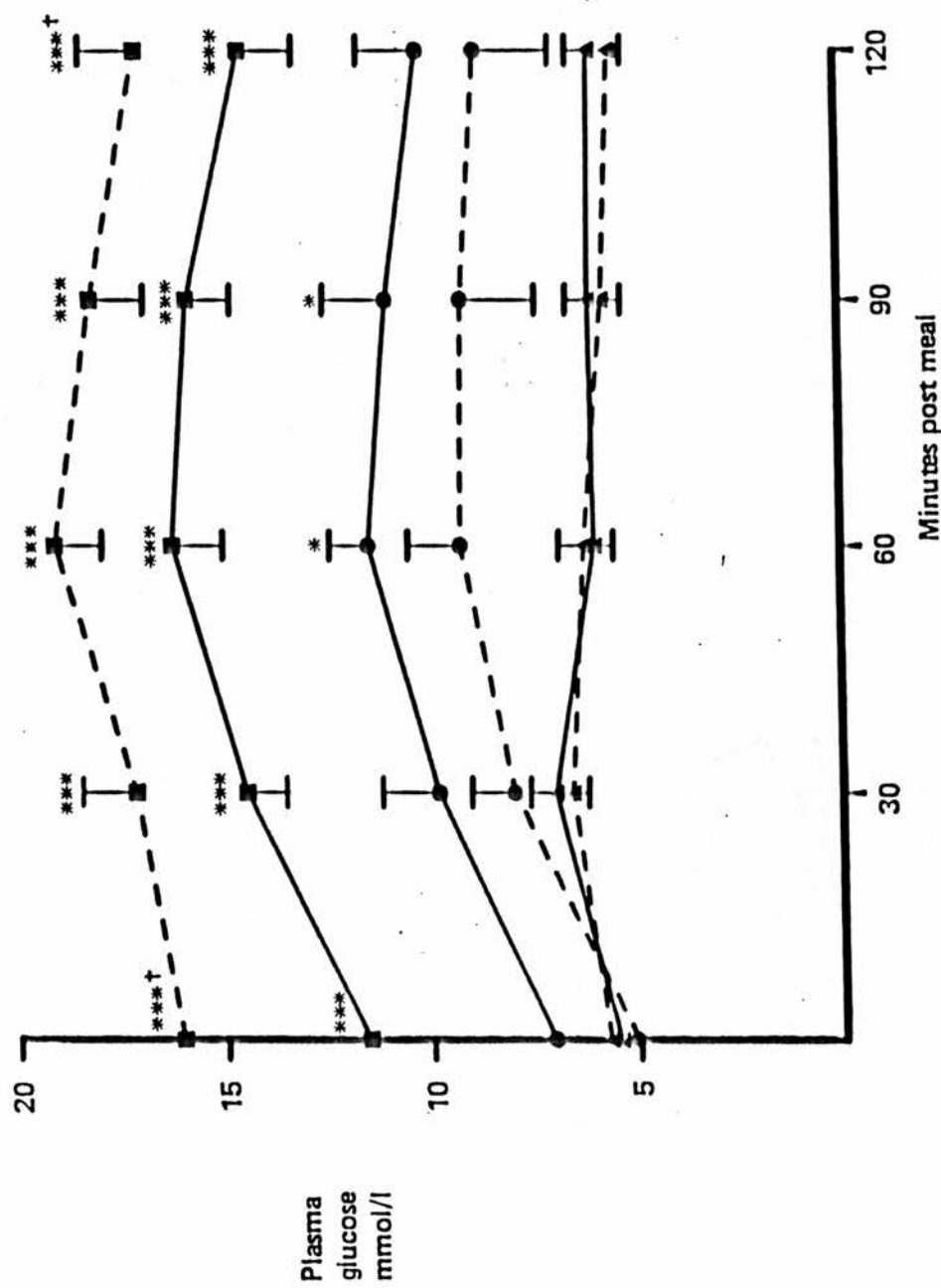


Fig. 3.6 Plasma glucose response to meal

Diabetic CT (■); Diabetic CSII (●); Non diabetic subjects (▲);  
 (—) normal diet; (---) fat supplemented diet. Mean  $\pm$  SEM.  
 \*Denotes comparison between diabetic and non diabetic subjects; \* $p < 0.05$ ; \*\*\* $p < 0.001$   
 +Denotes comparison between subjects on normal diet and fat supplemented diet; + $p < 0.05$ .

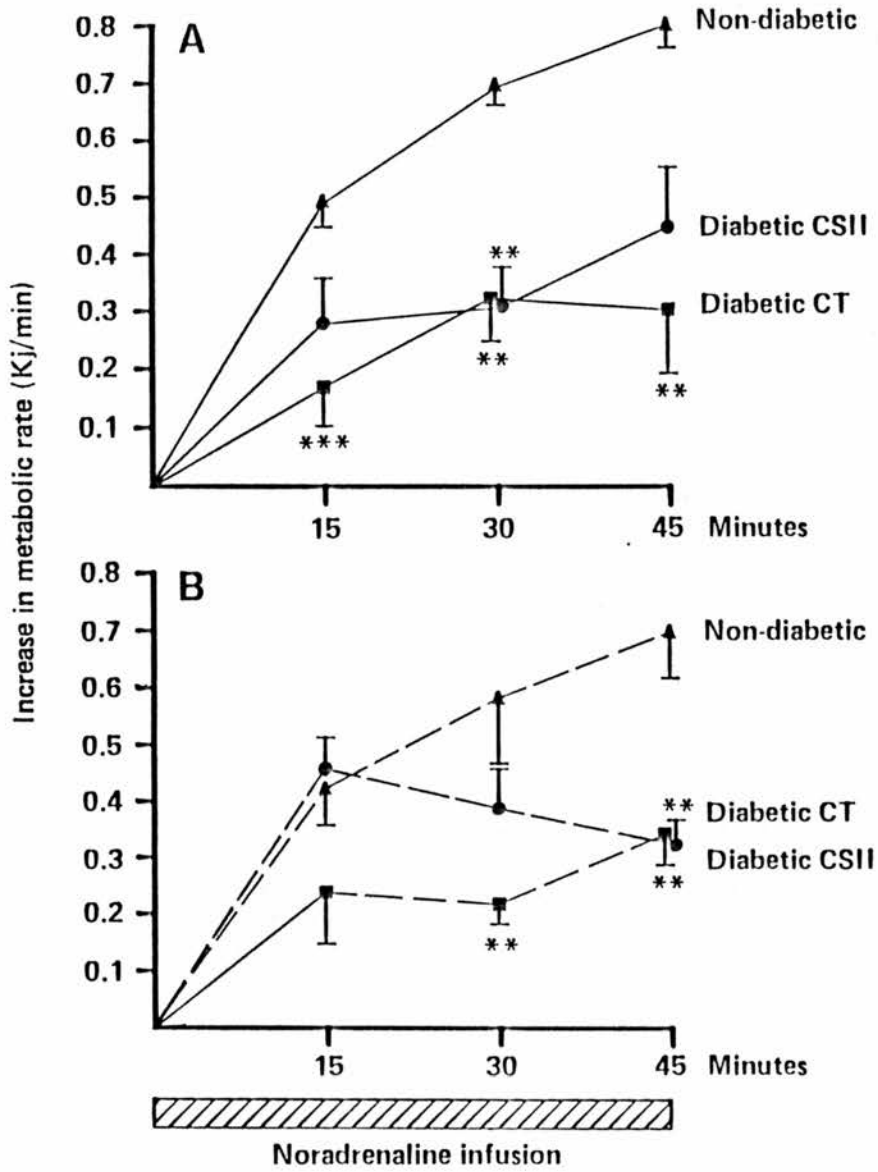


Fig. 3.7. Thermic response to noradrenaline infusion before (A) and during fat supplementation (B) in 8 non diabetic subjects (▲) and in 7 diabetic subjects on conventional therapy (■; CT) and on continuous subcutaneous insulin infusion (●; CSII). Diabetic vs non diabetic; \*\*p<0.02, \*\*\*p<0.01 Mean  $\pm$  SEM.

inaccurate assessment of the thermic response which was almost 50% greater than the normal response in the control subjects (41.8 KJ compared to 27.1 KJ). Noradrenaline infusion was well tolerated by all other subjects.

The incremental thermic response shown by the remaining diabetic subjects on CT was 54% lower than that shown by the control group and this blunting was reproduced both before and during fat supplementation. Further, the thermic response did not improve on CSII and remained 46% lower than that in controls. When repeated during fat supplementation there was no significant alteration in the response observed in any of the groups (Fig. 3.7 and Table 3.6).

### 3.3.7. Biochemical Response to Noradrenaline

#### 3.3.7.1. Glucose

Noradrenaline infusion resulted in a significant rise in mean plasma glucose at 15, 30 and 45 minutes in the control subjects. There was also a rise in mean plasma glucose in the diabetic subjects but this did not reach statistical significance (Fig. 3.8). As subcutaneous insulin continued to be absorbed (CT) and infused (CSII) the response was more variable, with one or two individual subjects in each group demonstrating a net fall in blood glucose during infusion. There were no significant differences when the rise of glucose between the control and diabetic subjects were compared. Diabetic subjects on normal diet had a significantly greater rise in plasma glucose on conventional therapy than on CSII ( $p < 0.05$ ; Table 3.7).

#### 3.3.7.2. Free fatty acids

Basal values and the rise in the fatty acids in response to noradrenaline were similar in all groups of subjects studied both in normal diet and during fat supplementation (Fig. 3.9(a) and Table 3.7).

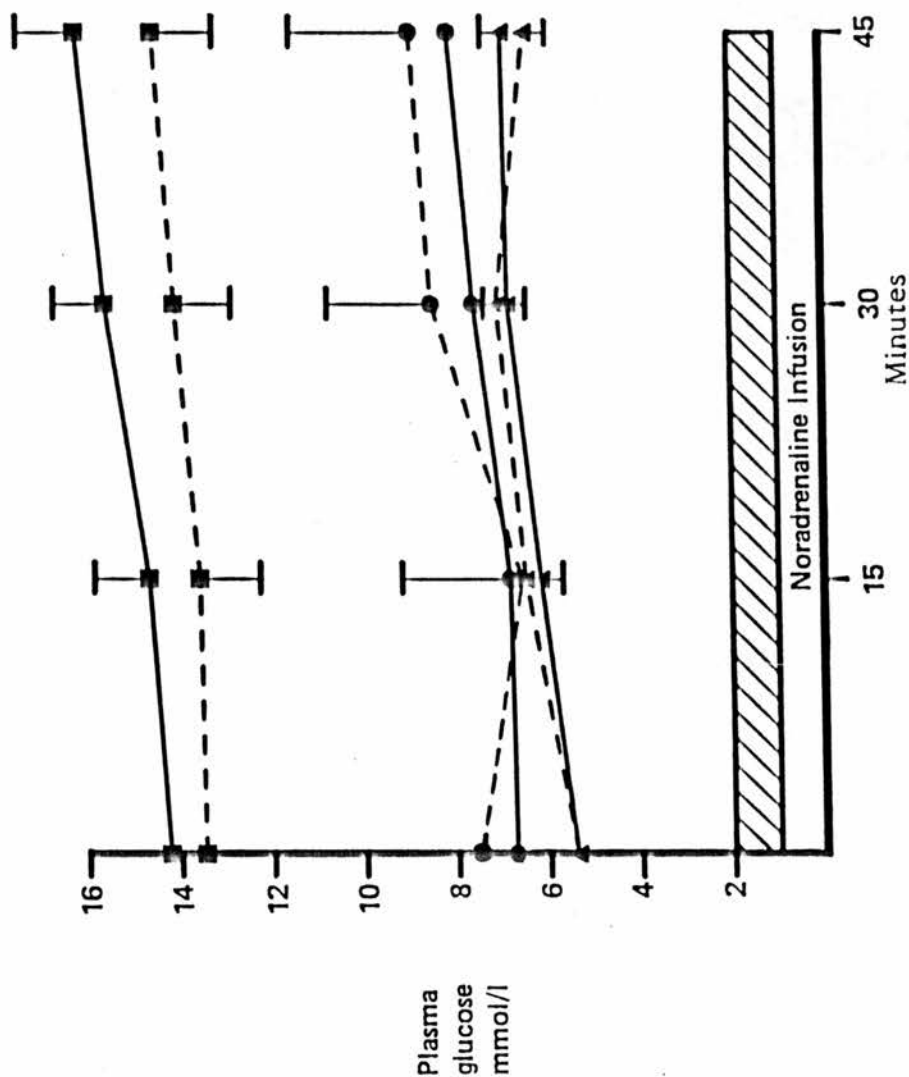


Fig. 3.8 Plasma glucose response to noradrenaline

Infusion before (—) and during (---) fat supplementation. Diabetic subjects on conventional therapy (■) had significantly greater plasma glucose than during CSII (●) or non diabetic controls (Table 3.7). There were no significant differences between diabetic subjects on CSII and non diabetic subjects (▲).

Subjects	Intake	Free Fatty Acids		Glycerol		Rise		Glucose		Rise
		Basal mmol/l	Rise mmol.min	Basal mmol/l	Rise mmol.min	Basal mmol/l	Rise mmol.min	Basal mmol/l	Rise mmol.min	
Non diabetic n = 8	Usual intake	0.381±0.036	23.1±2.3	0.133±0.045	4.64±0.34	5.5±0.1	43.1±6.0			
	Fat supplement	0.449±0.046	20.8±1.4	0.155±0.082	5.51±0.22	5.6±0.1	30.9±4.7			
Diabetic on CT n = 7	Usual intake	0.544±0.061	26.8±2.8	0.276±0.039***	5.35±0.64	14.1±1.6****	51.3±7.2 <sup>†</sup>			
	Fat supplement	0.477±0.041	23.1±2.9	0.344±0.073*	7.23±1.6	13.6±1.6****	40.9±15.0			
Diabetic on CSII n = 7	Usual intake	0.408±0.074	20.2±1.7	0.210±0.067	5.10±1.09	6.3±1.2 <sup>+++</sup>	17.8±14.7			
	Fat supplement	0.452±0.067	20.5±2.1	0.267±0.085	3.35±0.65	7.5±1.6 <sup>Δ</sup>	20.9±7.1			

Table 3.7: Biochemical response to noradrenaline infusion.

Mean ± SEM.

CT = Conventional therapy.

CSII - Continuous subcutaneous insulin infusion.

Diabetic on CT or CSII vs non diabetics (comparing usual intake vs usual intake, fat supplement vs fat supplement); \*p<0.05, \*\*\*p<0.01, \*\*\*\*p<0.01.

Diabetic on CSII on usual intake vs diabetic on CT on usual intake; <sup>†</sup>p<0.05, <sup>+++</sup>p<0.01.

Diabetic on CSII on fat supplement vs diabetic on CT on fat supplement; Δ p<0.01.

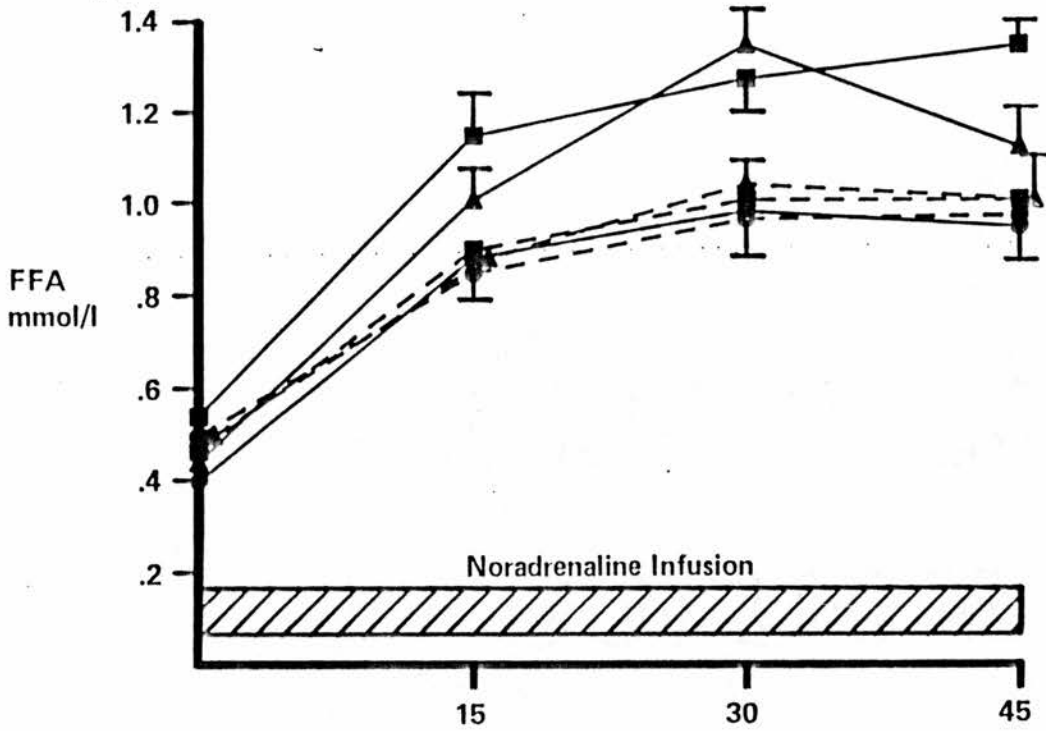


Fig. 3.9 (overleaf)



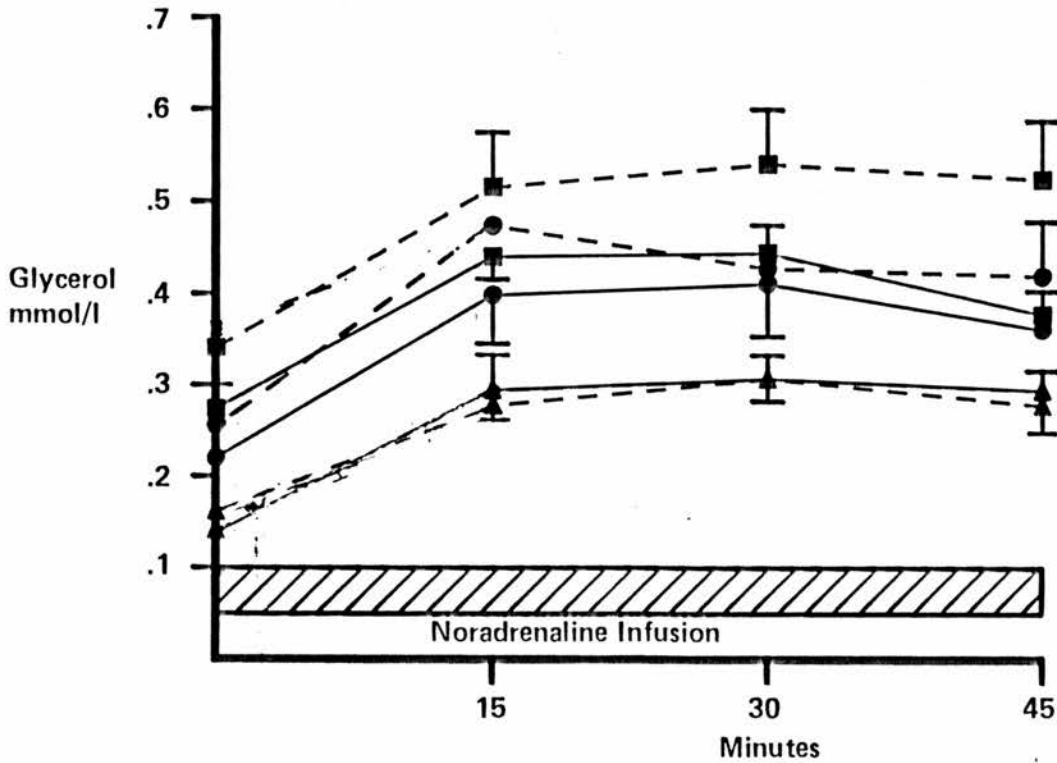
### FFA DURING NORADRENALINE INFUSION $\pm$ SEM

Fig. 3.9(a)



### GLYCEROL DURING NORADRENALINE INFUSION $\pm$ SEM

Fig. 3.9(b)



### 3.4. DISCUSSION

#### 3.4.1. CSII

Intensification of physician-patient contact, the increase in practical knowledge of the tripartite relationship between diet, insulin and exercise and the delivery of insulin via continuous subcutaneous insulin infusion all combined to achieve the goal of marked improvement in glycaemic control in this study approaching the non-diabetic state.

As this study was not designed to examine the efficacy of CSII as a method of insulin delivery compared to conventional therapy it is unfair to attempt to draw comparisons from the limited experience resulting from these few highly motivated subjects to the result of other studies comparing CSII to CT.

However, some broad conclusions can be drawn. The three major complications of CSII - cutaneous, hyperglycaemia and hypoglycaemia - have all been highlighted in other studies.

Cutaneous complications at the site of needle insertion have been well described (Pietri and Raskin, 1981; Levandowski et al., 1982). As infection at the site of insulin injection on CT is extremely rare, the report in one major study involving 161 patients of infected needle sites occurring in 29% of patients is of concern (Meckelenberg et al., 1984).

In the present study the clinical impression was that as the subjects became more familiar with the procedures involved in CSII, "short cuts" in aseptic technique and cannula care became more apparent. Although this has not led to local abscess formation, it has certainly contributed to an increase in the number of episodes of hyperglycaemia due to line blockage or disconnection, omission of meal time boluses and failure to check the amount of insulin in the syringe or the state of charge of the battery.

No episodes of frank ketoacidosis have been recorded in the remaining eight patients on CSII; other workers have estimated the incidence of this complication at one episode per 78 patient months (Meckelenberg et. al., 1984) to one episode per 32 patient months (Peden et. al., 1984). The lack of experience of ketoacidosis in the present study once again probably reflects the small numbers monitored intensively over a relatively short period of time.

The other major problem is hypoglycaemia. In several studies overt hypoglycaemia occurred no less frequently on CSII compared to CT (Kroc Collaborative Study Group, 1984; Lauritzen et. al., 1983; Home et. al., 1982). In the largest reported series of patients treated long term with CSII hypoglycaemic coma occurred with a frequency of one episode per 175 patient months and was slightly less common than in a group of 165 patients on conventional therapy (Meckelenberg et. al., 1984).

The three episodes of hypoglycaemic coma encountered in the short period of the present study contrasts with this experience but the sample size and duration of treatment obviates the drawing of conclusions.

Since the report of Steele and West (1985) which illustrated the potential deficiencies of an insulin regimen based solely on short acting insulin during pregnancy and the observations of Knight et. al. (1985) of the rapidity at which acute metabolic decompensation can occur on CSII when insulin supply is interrupted would make the author reluctant to expose a diabetic woman to the risks of CSII during pregnancy in future. The mild macrosomia of the infant of subject No. 5 despite adequate glycaemic control, as evidenced by HbA<sub>1</sub> estimation, may be a reflection of minor episodes of loss of control due to transient interruption of insulin delivery.

As evidence accumulates that similar levels of glycaemic control can be achieved using an intensified regimen based on long acting insulin with pre-prandial injections of short acting insulin (Calabrese et. al., 1982) without the risk of acute metabolic decompensation due to mechanical failure of insulin delivery, the practical role of CSII in the management of the Type I diabetic subject likely to emerge is that of a useful adjunct to achieve periods of optimal control in motivated patients under intense supervision.

In conclusion, although CSII is a highly attractive and convenient method of insulin delivery from the patients' point of view, in terms of its initial aim of achieving long term (i.e., over a period of 20-30 years) euglycaemia, its role remains limited.

#### 3.4.2. Resting Metabolic Rate

Quantitatively the RMR accounts for 65 - 75% of man's total 24 hour energy expenditure (Jequier, 1984). Hence relatively small changes in the RMR may have a substantial accumulative effect on long term energy balance.

One can calculate from the present study that those on CSII had a reduction in RMR amounting to the equivalent of 346 KJ (83 Kcal) per 24 hours. This reduction in RMR occurred despite a significant increase in body weight, which would itself have the effect of elevating the RMR, thus making this trend even more significant. If dietary intake had remained constant then this amount of energy would have been available for storage hence predisposing to weight gain. Together with the reduction in energy "loss" due to glycosuria this may partly explain the significant gain in weight that occurred in the diabetic subjects during CSII despite similar insulin dosage.

Home et. al. (1982) have also observed this phenomenon on CSII and in the 10 subjects studied weight significantly increased from a mean of 67.6 kg to 69.1 kg after six weeks of CSII and remained significantly elevated for the duration of the study. This reduction in RMR on CSII does not seem to be an adaptive process occurring over a period of time as the subjects on CSII showed a striking rise in RMR following the overnight withdrawal of insulin, thus suggesting that the restoration of a euglycaemic state is the major determinant of the abolition of this abnormality in Type I diabetic subjects. It is interesting to note, however, that this phenomenon was not noted in streptozotocin induced diabetes in rats. Rothwell and Stock (1981) found similar resting oxygen consumption in diabetic ( $13.3 \pm 0.3$  ml/min/W<sup>0.75</sup>) and non-diabetic ( $12.8 \pm 0.5$  ml/min/W<sup>0.75</sup>) rats.

An elevation of RMR on acute insulin withdrawal in already poorly controlled Type I subjects has also been shown by Nair and colleagues (1984). In this study hyperglycaemia (mean plasma glucose 19.3 mmol/l) associated with mild acidosis resulted in an elevation of the RMR 18% above that observed in the same subjects when euglycaemia was achieved (mean plasma glucose 5.8 mmol/l) using intravenous insulin.

The important point however arising from the present study is that the observed elevation of the RMR performed under the Harrow group's laboratory extremes of hyperglycaemia and euglycaemia is also observed in Type I diabetic subjects at levels of glycaemic control commonly encountered in clinical practice.

The mechanisms responsible for the increase in the RMR remain unclear. The metabolic acidosis associated with the uncompensated diabetic states cannot completely explain the increase in energy expenditure as in the study of Nair et. al. (1984), even those subjects who had a normal serum bicarbonate concentration had an elevated RMR.

As demonstrated in the present study with the infusion of noradrenaline, catecholamines can exert considerable influence in the RMR in man. Although diabetic ketoacidosis is associated with an elevation of these circulating hormones (Schade and Eaton, 1979), Alberti et. al., (1975) demonstrated that withdrawal of insulin for 41 hours was not associated with a significant elevation of catecholamines. These hormones are therefore unlikely to be making a major contribution to the observed elevation in RMR.

The other major hormonal insulin antagonists which may be elevated during poor diabetic control are cortisol and growth hormone (Schade and Eaton, 1979). Nair et. al. (1984) however found no significant correlation with these hormones and energy expenditure in their study. A significant correlation however was found with plasma glucagon concentration but this does not prove that glucagon is responsible for the raised energy expenditure and may merely represent a secondary phenomenon.

The biochemical pathways determining rates of fat, carbohydrate and protein synthesis and oxidation and the complex cycling of substrates from one pathway to another constitute a major component of resting energy expenditure.

The Harrow Group (Nair et. al., 1983b) has demonstrated increased rates of both protein synthesis and degradation during poor diabetic control and have more recently (Nair and Halliday, 1985) demonstrated a positive correlation between the rate of protein synthesis and energy expenditure in non-diabetic subjects. This may be one of the factors contributing to the rise in RMR.

An increased basal rate of lipolysis, and subsequently fat oxidation, is suggested in the poorly controlled diabetic subjects by the significantly greater concentrations of fasting plasma glycerol and the tendency, which

did not reach statistical significance ( $p = .06$ ), for a lower respiratory quotient on CT ( $0.77 \pm .02$ ) which reverted to normal on CSII ( $.83 \pm .02$ ). This factor too may also contribute to the elevation in RMR.

The final factor which should be considered is the energy expenditure involved in the increased rate of gluconeogenesis in poorly controlled subjects (De Fronzo et. al., 1985).

Thus the significant elevation in RMR observed in the poorly controlled diabetic state may be due to a number of factors and if problems of weight gain on improvement of glycaemic control are to be avoided appropriate dietary advice should be instituted.

#### 3.4.3. Thermic Response to the Meal

The thermic effect of food makes a significant contribution to the remaining 20-35% of total daily energy expenditure.

In the present study there was no significant difference in the thermic response to the mixed liquid meal when either the diabetic or non-diabetic subjects were ingesting their normal diet irrespective of the degree of glycaemic control.

If insulin has a role to play in determining the thermic response to a physiological mixed meal one would have expected the hyperglycaemic (and thus relatively insulin deficient) subjects on CT to have a lower response than during CSII and when compared to non-diabetic controls. The present results, however, are in contrast to the work of Golay and colleagues (1982)

who demonstrated a progressive diminution of the thermic response to a 100 g oral glucose load with increasing insulin resistance (Fig. 1.4(b)).

The concurrent finding of blunted thermic responses to glucose loads (Pittet et. al., 1976; Schutz et. al., 1984b), high protein meal (Kaplan and Leveille, 1976), fat (Swaminathan, 1985) and mixed meals (Shetty et. al., 1981; Bessard et. al., 1983; Schutz et. al., 1984a) in obese subjects has led to the hypothesis that insulin acts as an important mediator of these abnormalities in the predisposition of some individuals to obesity (Felig, 1984).

An almost equal number of other workers however have failed to demonstrate such abnormalities in obese subjects (Bradfield and Jourdan, 1973; Sharief and Macdonald, 1982; Nair et. al., 1983; Welle and Campbell, 1983a; Swaminathan et. al., 1985).

These conflicting findings may be due to a number of factors including the calorimetric method used, the duration of study and different composition of the test meal. Jequier (1984) has recently demonstrated that these abnormalities may only occur in subgroups of obese subjects and therefore the individual subjects studied provide another variable in this field of conflicting data.

To return to the role of insulin however, close perusal of the literature might suggest that the data from the present study are not incompatible with the previous findings in obese and Type II diabetic subjects. Firstly the work of Golay et. al. (1982); Pittet et. al. (1976) and Schutz et. al. (1984b) was concentrated solely on the thermic response to an unphysiological stimulus of 100 g or 50 g oral glucose loads. As diminished glucose oxidation is one of the cardinal features of insulin resistant states (Felber et. al., 1981), a diminution of the thermic response is thus not surprising. When the data of Golay et. al. (1982) (Fig. 1.4(b)) is closely



examined the differences between the grossly obese subjects with impaired glucose tolerance and Type II diabetic subjects characterised by an exaggerated insulin response and the non-obese subjects reduces from a significance of  $<0.05$  and  $<0.001$  respectively to statistically indistinguishable from lean controls when the correction for unmetabolised glucose is made. Pittet's data documents a 40-45% reduction in the rate of carbohydrate oxidation in the obese group compared to controls. The data of Schutz et. al. (1984b) is also hard to reconcile with a primary role for insulin in the generation of the thermic response as when the obese subjects were studied after weight loss the corrected thermic response to glucose, expressed as a percentage of the energy load, fell further from 6.2% to 3.4% despite return of glucose tolerance to normal. The same group have also demonstrated that the apparent diminution of the thermic response to infused glucose can be corrected by establishing similar rates of cellular glucose uptake using the euglycaemic hyperinsulinaemic clamp technique (Ravussin et. al., 1985).

When studies using a more physiological meal are examined a similar pattern emerges - the persistence of thermic abnormalities in obese subjects despite the return of insulin sensitivity is indistinguishable from that of lean controls when weight loss is achieved (Shetty et. al., 1981; Bessard et. al., 1983).

Thus from the present study it is likely that the absence of a demonstration of a thermic abnormality in the diabetic subjects was due to a number of factors. Firstly despite the five-fold greater rise in plasma glucose following the meal, indicative of unmetabolised glucose, this alone was not of sufficient magnitude to cause a significant diminution of the total thermic response resulting from the processing of all the constituents of the mixed meal.

Secondly, when the literature is examined critically concerning thermic abnormalities to food in obese and Type II diabetic subjects it must be concluded that these abnormalities are only indirectly mediated in insulin.

#### **3.4.4. Thermic Response to Noradrenaline**

The thermic response to noradrenaline also contributes to the remaining 24 hour energy expenditure and although the magnitude of this component is difficult to precisely quantify as it fluctuates according to the ambient sympathetic tone, at least 5% and up to 10% of total daily energy expenditure may be accounted for by this component.

In the present study the thermic response to noradrenaline was reduced by over 50% in the diabetic subjects irrespective of their degree of glycaemic control. Although the total daily energy "deficit" due to these blunted responses cannot be calculated from the present study, one can speculate that it is unlikely they are of sufficient magnitude to outweigh the effect of the raised RMR in poorly controlled diabetes mellitus. However, on attainment of optimal glycaemic control these blunted responses would be one further factor predisposing to weight gain if dietary intake remained unchecked.

What is the mechanism of this thermogenic abnormality in the Type I diabetic subjects?

Firstly, a consideration of the animal models in which blunted noradrenaline responsiveness has been associated with diabetes would suggest that altered insulin sensitivity in the diabetic subjects might be a contributory factor. As reviewed in Section 1.3.3.4, Rothwell and Stock (1981) demonstrated a requirement for insulin in the thermogenic adaptation to cafeteria feeding in rats. Of particular interest in view of the results of

the present study, they also demonstrated that the thermic response to noradrenaline in poorly controlled streptozotocin diabetic rats on normal stock diet (blood glucose  $19.2 \pm 1.0$  mmol/l) was blunted by over 50% (rise in oxygen consumption  $3.3 \text{ ml/min } W^{0.75}$ ) compared to non-diabetic rats ( $7.6 \text{ ml/min/W}^{0.75}$ ). However following insulin replacement (blood glucose unspecified) the thermic response returned to normal ( $6.45 \text{ ml/min/W}^{0.75}$ ). This suggested a direct requirement for insulin not only in the adaptive response to cafeteria feeding but also in the normal thermic response to noradrenaline on normal diet. This contrasts with the persistent blunting of noradrenergic thermogenesis despite insulin replacement using CSII in the present study. Cunningham et. al. (1983) and Seydoux et. al. (1983) also demonstrated abnormalities of brown adipose tissue metabolism in diabetic rats and the results of the former workers demonstrated that these also predisposed to weight gain.

When studies reflecting noradrenaline responsiveness and insulin sensitivity in man are considered similar conflicting results are found. The presence (Jung et. al., 1979) and absence (Katzeff et. al., 1986; Finer et. al., 1985) of blunted thermic responses to noradrenaline infusion in obese hyperinsulinaemic subjects have both been documented. In all these studies the subjects were also examined following significant weight loss and there was no improvement (Jung et. al., 1979), no change (Katzeff et. al., 1986) and in fact a blunting of the response (Finer et. al., 1985) on the attainment of significant weight reduction. Jung et. al. (1979) hypothesised from their findings that the thermic defect to noradrenaline was an inherent abnormality in the obese state whereas Finer's group attributed the blunting to their extreme hypocaloric (390 kcal; 1.6 MJ/day) diet. With the recent finding that blunting the thermic response to noradrenaline in lactating subjects (Illingworth et. al., 1986) was associated with increased insulin

sensitivity during lactation the case for a simple relationship between noradrenergic thermic responsiveness and insulin sensitivity becomes less tenable. Further, Bazelmans et. al. (1985) failed to find any correlation between insulin sensitivity and noradrenaline flux in obese subjects before and after weight loss.

Thus, when the evidence from human studies is examined critically the hypothesis proposed by Rothwell and Stock (1981) for a significant thermogenic role for insulin is not supported in man as these thermic abnormalities have been demonstrated in both increased (lactation), normal (post-obese) and decreased (obese) states of insulin sensitivity.

The findings from the present study, which demonstrated that despite marked changes in glycaemic control persistence of thermic abnormalities occurred, would tend to indicate that other mechanisms than solely insulin are concerned.

At this point it is worth examining the evidence for changes in insulin sensitivity during CSII for although I have demonstrated a marked improvement in glycaemic control on CSII on similar dosage of insulin, this does not necessarily infer an improvement in insulin sensitivity.

Initial reports suggested that the insulin resistance associated with Type I diabetes (Del Prato et. al., 1983) was reversible on improvement of glycaemic control with CSII (Lager et. al., 1983). However a review of the current literature demonstrates that although some workers have demonstrated an improvement of insulin mediated glucose disposal during CSII (Lager et. al., 1983; Simonson et. al., 1985; Beck-Nielson et. al., 1984; Yki-Jarvinen et. al., 1984), others have failed to do so (Simonson et. al., 1982). The values for glucose disposal however remain at best 25% lower (Lager et. al., 1983) in Type I diabetic subjects on CSII than that of the respective non-diabetic control group (Table 3.8).

<u>Study</u>	<u>Duration of CSII</u>	<u>Glucose Disposal Rate (mg/kg/min)</u>		
		<u>Diabetic CT</u>	<u>Diabetic CSII</u>	<u>Non Diabetic Controls</u>
Lager et. al. (1983)	9 days	4.3 ± 0.4**	6.0 ± 0.7 <sup>++</sup>	8.0 ± 0.5
Beck-Nielson et. al. (1984)	6 months	4.3 ± 0.61**	7.5 ± 1.06 <sup>+++</sup>	11.5 ± 1.2
Yki-Jarvinen et. al. (1984)	6 months	4.65 ± 0.41***	5.90 ± 0.60 <sup>+</sup> *	7.20 ± 0.42
Simonson et. al. (1985)	6 months	3.92 ± 0.36	5.33 ± 0.75 <sup>+</sup> **	7.03 ± 0.22

Table 3.8: INSULIN RESISTANCE DURING CSII.

Diabetic vs control \*\*\*p>0.001, \*\*p>0.01, \*p<0.05. Insulin resistance measured as glucose disposal rate (mg/kg/min) using the euglycaemic clamp technique in diabetic subjects on conventional therapy (CT); the same subjects during treatment with continuous subcutaneous insulin infusion (CSII) and compared to matched non diabetic controls<sup>+++</sup> p>0.0125; <sup>++</sup>p>0.05; <sup>+</sup>p>0.01.

To further examine the role of insulin resistance in determining the blunted thermic responses to noradrenaline a further experiment was attempted.

The biguanide derivative, metformin historically has been used in addition to insulin therapy in poorly controlled Type I diabetic subjects in attempts to reduce insulin dosage (Duncan and Seaton, 1962). More recently (Gin et. al., 1985) metformin has been shown to increase insulin mediated glucose disposal in Type I diabetic subjects suggesting that this agent may be used to pharmacologically decrease insulin resistance. Six of the diabetic subjects in the present study, after completion of the protocol agreed to take metformin 500 mg three times daily with meals for fourteen days, whilst receiving continuous subcutaneous insulin infusion. Only three of the subjects however managed to complete fourteen days treatment, the others developing nausea and abdominal upset necessitating withdrawal of metformin. In these subjects the thermic response to noradrenaline was measured before and on the fourteenth day of metformin therapy. Metformin improved the thermic response to noradrenaline in all three subjects as shown below.

<u>Subject No.</u>	<u>CSII Alone</u>	<u>CSII + Metformin</u>
1	14.9	23.0
2	3.2	11.4
7	6.3	19.5

Thermic response to noradrenaline (KJ) on CSII and with the addition of metformin 500 mg t.d.s.

Although insulin mediated glucose disposal was not assessed before and during metformin therapy it is interesting to speculate whether a change in insulin sensitivity might be implicated in the improvement of the thermic

response. In contrast, metformin does not increase the thermic response to noradrenaline in Type II diabetic subjects (Section 4.3.6.) but these subjects already had a normal thermic response to this catecholamine prior to metformin therapy. Thus, although studies such as this will need to be extended before any firm conclusions can be drawn, perhaps these results give some credence to the past practice of administering biguanide to obese subjects poorly controlled on insulin (Duncan and Seaton, 1962).

Therefore, if insulin is not a major determinant of these altered thermogenic responses in the present study what other factors may be involved?

As about 50% of the thermic response effect of noradrenaline is related to lipolysis and fat oxidation (Havel et. al., 1964) a diminution of the lipolytic response to catecholamines in the obese, lactating and the present diabetic subjects might explain the differing thermic responses. Conversely, an insulin resistant state in obesity or poorly controlled diabetes should predispose to enhancement of the thermic response by enhancing lipolysis. However, the obese (Jung et. al., 1979), lactating (Illingworth et. al., 1986) and the Type I diabetic subjects demonstrated similar rates of lipolysis despite marked blunting of the thermic responses.

Noradrenaline infusion has been employed as a model for non-shivering thermogenesis and brown adipose tissue metabolism in man (James and Trayhurn, 1981). The hypothesis that these thermic differences are due to diminished brown adipose tissue activity in adult man are discussed in Chapter V.

Recently the work of Astrup et. al. (1985a) in man has demonstrated that chronic administration of the sympathomimetic agent, ephedrine, may enhance thermogenesis. 50% of this heat production appears to be generated in skeletal muscle (Astrup et. al., 1985b), with a negligible contribution from



perirenal brown adipose tissue and may involve activation of  $\beta_2$  receptors (Fagher et. al., 1986). Substrate cycling and kinetics within the metabolic masses of skeletal muscle and liver (Berry et. al., 1985) may partly account for the differences noted by the differential shunting of substrates to either energetically efficient or wasteful pathways in different physiological (lactation) or pathological (obesity and diabetes mellitus) states.

Once the precise biochemical mechanisms and the tissue or tissues involved in the generation of the normal thermic response to noradrenaline are elucidated then and only then will the mechanism and significance of the blunted responses to catecholamines be fully understood.

#### **3.4.5. Effect of Fat Supplementation**

Since Neumann demonstrated in 1902 the ability to maintain his body weight within narrow limits despite increasing his daily energy intake from 7.4 MJ to 10 MJ over a period of 3 years, the elusive concept of "luxuskonsumption" has been investigated by many groups. Although almost all studies agree on the finding of an elevation of the RMR during overfeeding (Section 1.4.1) opinion is divided as to whether there is evidence for an adaptive mechanism for the dissipation of excess calories as heat in man.

In the present study there was no demonstrable change in the RMR or thermic response to noradrenaline in either the diabetic or control groups during fat supplementation. The study design resulted in an approximately 30 - 50% increase in energy intake mainly in the form of fat which was sufficiently acceptable to assure compliance and not lead to deterioration in control in the diabetic subjects over a short period of time. It is likely, however, in retrospect, that the duration of fat supplementation was insufficient to demonstrate a significant change in energy expenditure, but



does demonstrate the inability of a group of individuals to rapidly compensate for an increase in energy intake by a measurable intake in energy expenditure. Dalosso and James (1984) in a meticulously controlled study using 24 hour whole body indirect calorimetry and accurate (to within 1 g) weighed intakes, overfed eight men with a similar amount of fat as used in the present study (5 MJ) for a period of one week. Their results however demonstrated only a small thermogenic component in excess of that predicted from the theoretical energy cost of fat storage and illustrate the difficulties involved in the accurate assessment of regulatory thermogenesis in man.

The apparent blunting of the thermogenic response to food in the poorly controlled diabetic subjects following fat supplementation resulted in a diminution of energy expenditure in the region of 30 KJ (7 kcal) in the 2 hour period. Although post-prandial thermogenesis may continue for up to 24 hours following ingestion this energy deficit may have been appreciably greater if we had measured the expenditure resulting from three main meals and associated snacks over a 24 hour period using direct calorimetry.

The mechanisms for this blunting in the second hour cannot be conclusively determined from this study. Two factors, however, may be significant. When the poorly controlled diabetic subjects were supplemented with fat, the mean blood glucose concentration fasting and during the post-prandial period tended to be 3 to 4 mmol/l higher compared to the same subjects when studied on normal diet and during CSII (Figure 3.6). Fat overfeeding may have induced a degree of insulin resistance (Olefsky, 1976) and thus resulted in diminished glucose oxidation.

The other factor that was not assessed in the present study was that the amount of unmetabolised glucose in the glucose space and the amount lost in the urine and thus unavailable for glucose oxidation was greater in the

fat-fed poorly controlled diabetic subjects. The energy deficit of 30 KJ would have to be accounted for by an urinary loss of glucose of 2 g in the 2 hour period.

#### 3.4.6. Conclusions

In conclusion, it appears that adequate glycaemic control with CSII does not correct all the metabolic abnormalities of Type I diabetes mellitus. These patients seem to be at a disadvantage regarding energy balance when glycaemic control is improved. In this setting the RMR increases appreciably and if a reduction in intake is not advised then weight increase is likely to ensue. From a wider viewpoint these data also suggest the role of insulin in the control of thermogenic responses to mixed meals and noradrenaline in man is a relatively minor one, in that apparently normal thermic responses to food occurred despite poor glycaemic control and conversely blunted thermic responses to noradrenaline occurred during optimal control on CSII.

Chapter IV

Energy Expenditure in Type II Diabetic Subjects  
on Oral Hypoglycaemic Therapy

## SUMMARY

The treatment of the obese diabetic subject with metformin tends to promote weight loss whereas treatment with sulphonylurea derivatives tends to result in weight gain.

In order to assess whether these changes in weight result from alterations in energy expenditure, the resting metabolic rate and the thermic responses to food and noradrenaline were measured in seven Type II diabetic subjects while on metformin and sulphonylurea therapy.

The resting metabolic rate and thermic responses were similar on metformin and sulphonylurea therapy and these responses did not differ significantly from that predicted for non-diabetic subjects.

It is concluded that Type II diabetic subjects do not exhibit any specific abnormality of energy expenditure while on therapy and that metformin does not facilitate weight loss by enhancing energy expenditure.

#### 4.1. INTRODUCTION

Obesity is one of the major factors in the aetiology of Type II diabetes mellitus and weight loss through carbohydrate and caloric restriction results in some improvement in glycaemic control in the majority of subjects. However, in clinical practice, many patients fail to achieve adequate weight loss and improvement of glucose tolerance despite alleged restriction of intake.

Controversy rages as to whether this is merely due to dietary indiscretion on the part of the subject or to whether there is an inherent abnormality of energy expenditure in these subjects which predisposes to failure of weight loss. The consistent demonstration of an elevation of energy expenditure in obese subjects compared to matched lean subjects when measured as the RMR (James et. al., 1978; Halliday et. al., 1979; Finer et. al., 1986) or as total daily energy expenditure (Bessard et. al., 1983; Prentice et. al., 1986) lends support to the concept of the "obese deceiver" (Southgate, 1986). In 1979, however, Keen et. al. documented an association, counter to expectation, of increased body mass index and glucose intolerance with decreased energy intake and hypothesised that a "low energy throughput state" may contribute to the pathogenesis of obesity and abnormal glucose tolerance. Other studies have demonstrated abnormalities of energy expenditure in the obese and Type II diabetic subjects in response to intravenous (Ravussin et. al., 1983) and oral (Golay et. al., 1982) glucose and it has been further suggested that these may predispose to failure of weight loss and relapse of the obese state (Schutz et. al., 1984b).

Nevertheless, when the overweight Type II diabetic subject fails to achieve adequate glycaemic control on dietary measures alone, treatment with oral hypoglycaemic therapy is indicated.

The biguanide derivative, metformin, is often considered to be the drug of first choice in the management of these subjects as this agent has been demonstrated to facilitate weight loss whereas the alternative therapeutic option, sulphonylurea therapy, usually predisposes to weight gain.

In 1968, Clarke and Duncan demonstrated in a prospective study involving 77 obese Type II diabetic subjects previously inadequately controlled on dietary restriction, a mean weight gain of 11.6 lb (5.3 kg) on sulphonylurea therapy in contrast to a significant mean weight loss of 2.7 lb (1.3 kg) on metformin during one year on each therapy (Fig. 4.1). Other workers have confirmed these findings with metformin (Pedersen, 1965; Mehotra and Young, 1967) and the other major biguanide derivative, phenformin (Patel and Stowers, 1964; Mirsky and Schwartz, 1966).

Nine years following their original observations Clarke and Campbell (1977) confirmed not only a similar pattern of weight loss and gain on metformin and sulphonylurea but also demonstrated that metformin was equally effective as chlorpropamide in achieving glycaemic control. Thus, metformin has emerged as the treatment of choice of the obese Type II diabetic subject who has failed to control on dietary measures alone. Biguanides have also been demonstrated in a placebo controlled study to induce a small change in weight in non-diabetic obese subjects (Munro et. al., 1969).

Despite the widespread agreement of these workers on the attainment of weight loss with metformin, opinions vary widely as to the mechanism whereby this is achieved. Some workers have attributed the reduction in weight to a diminution in energy intake as a result of the anorectic properties of biguanide therapy (Patel and Stowers, 1964) whereas others have failed to find such a relationship (Weller, 1965; Pedersen, 1965;

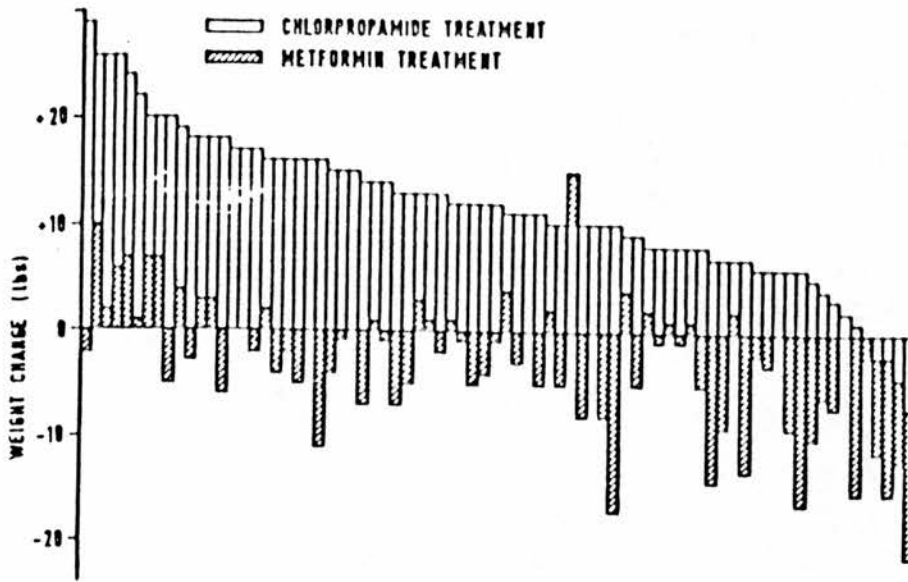


Fig. 4.1. Mean weight change (lbs) during twelve months treatment with chlorpropamide and with sulphonylurea ( $n = 77$ ).

Reproduced from Clarke and Duncan (1968) with the permission of the Lancet.

Mehotra and Young, 1967; Clarke and Duncan, 1968).

The other postulated mechanism of 'caloric sparing' by metformin is that of induction of malabsorption. Although the biguanide derivatives have been demonstrated to result in reduction of intestinal absorption of glucose (Biro et. al., 1961; Czyzyk et. al., 1967), amino acids (Caspary and Creutzfeld, 1973) and triglycerides (Curtis-Prior, 1982) in animal models and vitamin B12 in man (Tomkin et. al., 1971) there is no conclusive evidence that this can account for the therapeutic effect of metformin on weight control in diabetic man.

Clarke and Duncan (1968) over 18 years ago suggested that "if reduction in food intake is not the cause of obese diabetics tending to lose rather than gain weight during biguanide therapy then the net increase in retained calories must be disposed of by some catabolic non-tissue forming metabolic process". Despite this, the hypothesis that metformin may act to increase energy expenditure in these subjects has not been previously investigated. The present study was therefore designed to investigate whether alterations in energy expenditure could account for the differences in weight gain reported during metformin and sulphonylurea therapy.

## 4.2. EXPERIMENTAL PROCEDURE

### 4.2.1. Protocol

The RMR and thermic responses to a mixed meal and noradrenaline during treatment with metformin or a sulphonylurea derivative were compared in nine Type II diabetic subjects in an open crossover study.

The nine subjects, acting as their own control, were studied initially on their usual hypoglycaemic therapy. At recruitment three subjects were taking metformin and six subjects sulphonylurea derivatives. Those subjects



initially on sulphonylurea had their therapy changed to metformin 1500 mg daily (Lipha Pharmaceuticals, Ltd.) in divided doses with meals, whereas those subjects initially on metformin were changed to glipizide 5 mg daily (Pfizer, Ltd.).

The alternative therapy was continued for 15 days and measurements of energy expenditure were repeated on the 14th and 15th days following changeover. All subjects maintained their prescribed diet during the study.

Subjects were alerted to the potential adverse reactions of hypoglycaemia during sulphonylurea therapy and gastro-intestinal symptoms during treatment with metformin and were instructed to report these immediately.

#### 4.2.2. Subjects

Nine Type II diabetic subjects, aged less than 55 years, who had been treated with diet and oral hypoglycaemic therapy for at least three months were recruited from Ninewells Hospital Diabetic Clinic between April and December 1985. As the study involved infusion of noradrenaline, hypertensive subjects (sitting blood pressure of greater than 140/90 mmHg) were excluded. Concurrent medication with  $\beta$ -blockers, sympathomimetic agents and thyroxine, which may alter the RMR and thermic responses, contraindicated recruitment to the study. All subjects had normal renal, hepatic and thyroid function as estimated by measurement of plasma urea, creatinine, bilirubin, alkaline phosphatase, alanine and aspartate amino transferases, thyroxine and TSH concentrations. None of the subjects had evidence of complications of diabetes mellitus using the criteria described in Section 3.2.1.1.

Those subjects not already familiar with home blood glucose monitoring (BM Stix, Boehringer-Mannheim Ltd.) were instructed in this technique. Routine blood testing was performed twice daily (fasting and

2 hours following the largest meal of the day) during the two weeks prior to study on their usual hypoglycaemic agent and also during the two week period on alternative therapy. The age, sex, weight, body mass index, HbA<sub>1c</sub> % and oral hypoglycaemic therapy at recruitment of those subjects that completed the study are shown in Table 4.1.

#### **4.2.3. Body Weight**

Body weight was measured prior to the measurement of RMR on first study day, in light clothing without shoes, on each therapy.

#### **4.2.4. Measurement of Energy Expenditure**

Energy expenditure was measured using the indirect ventilated hood calorimeter described in Section 2.1.2 and all subjects were familiarised with the apparatus prior to study.

##### **4.2.4.1. Resting Metabolic Rate**

The resting metabolic rate was measured following an overnight fast and prior to the measurement of the thermic effect of a meal or noradrenaline on both metformin and sulphonylurea therapy, according to the protocol described in Section 2.1.3. All measurements were performed prior to the ingestion of the morning dose of the oral hypoglycaemic agent.

##### **4.2.4.2. Thermic Response to the Meal**

A similar liquid meal to that described previously (Section 2.1.5.1) was given to each subject following the measurement of the RMR. The oral hypoglycaemic agent under study was taken at the start of each meal. Venous blood samples were withdrawn for analysis of glucose and insulin concentrations at -5, 0, +30, +60, +90 and 120 minutes post ingestion and

Subject	Age (years)	Sex	Weight (kg)	Body Mass Index (kg/m <sup>2</sup> )	HbA <sub>1c</sub> %	Duration of Diabetes (years)	Dose of oral hypoglycaemic agent (mg/day)
1	41	M	102.6	32.0	5.9	2	Metformin 1500
2	44	M	121.1	37.9	10.8	2	Glibenclamide 20
3	46	M	66.6	22.3	9.6	1.5	Chlorpropamide 500
4	52	F	73.8	27.8	7.2	2	Chlorpropamide 200
5	42	F	71.3	26.2	10.9	0.5	Gliclazide 160
6	44	F	104.7	35.8	9.6	2	Metformin 1500
7	48	M	87.5	27.3	6.4	1	Chlorpropamide 300
Mean	45.3	M:F	89.7	29.9	8.62	1.57	
± SD	3.7	4:3	20.4	5.6	2.1	0.6	

Table 4.1: Subject details of those who completed study protocol

were collected and prepared in a similar manner to that described in Section 2.2.1.

#### 4.2.4.3. Thermic Response to Noradrenaline

Noradrenaline was infused at a dose of 0.1 µg/kg IBW/min as previously described (Section 2.1.5.2) after the measurement of the RMR and prior to the morning dose of oral hypoglycaemic therapy. Venous blood samples were obtained at -5, 0, +15, +30 and +45 minutes during infusion for analysis of free fatty acids, glycerol, glucose and insulin concentrations.

#### 4.2.5. Plasma Insulin

Plasma insulin was measured using a polyethylene glycol (PEG) assisted double antibody technique (Insulin ( $^{125}\text{I}$ ) KIT, Cambridge Medical Diagnostic Inc.). The principle depends on the competition between insulin in the plasma and a fixed amount of radiolabelled ( $^{125}\text{I}$ ) insulin for a limited number of binding sites on an antibody raised in guinea pigs against insulin. The proportion of bound radiolabelled insulin varies inversely with the concentration of unlabelled insulin in the plasma sample. Precipitation of the antigen-antibody complexes is accomplished by a combination of goat anti-guinea pig gamma globulin and PEG. This insoluble complex is separated by centrifugation at 1500 G for 10 minutes. The quantity of unlabelled antigen in an unknown sample is thus determined by comparing the radioactivity of the precipitate after centrifugation with values established using known standards (5-300 mU/l) in the same assay system. The inter-assay C.V. was 8.4% and intra-assay 6.9%.

## 4.3. RESULTS

### 4.3.1. Efficacy of Alternative Hypoglycaemic Therapy

#### 4.3.1.1. Adverse Reactions

Two subjects failed to complete the study protocol due to adverse reactions on alteration of therapy. One subject was withdrawn from the study after 5 days of glipizide therapy as he complained of symptoms of headache and sweating 2 to 3 hours following meals associated with BM Stix values of between 2 to 4 mmol/l. Glipizide was therefore discontinued and he was recommenced on dietary treatment alone.

Another subject developed abdominal cramps and watery diarrhoea subsequent to changing to metformin therapy which persisted after 4 days of therapy. Although this may have responded to reduction in the dosage of metformin, he was withdrawn from the study and recommenced chlorpropamide.

Of the remaining five subjects who changed from sulphonylurea to metformin therapy, two (subjects Nos. 2 and 4) noted mild looseness of their bowel motions which resolved spontaneously after 3 to 4 days without alteration of the dosage of metformin.

#### 4.3.1.2. Glycaemic Control

In the remaining 7 subjects who successfully completed the study protocol, glycaemic control was similar on metformin and sulphonylurea therapy. This was estimated not only from the subjects' own records of home glucose monitoring which ranged from 4 to 10 mmol/l but also from the fasting plasma glucose concentrations obtained prior to the meal and noradrenaline infusion on both treatments (Table 4.2).

NORADRENALINE INFUSION							MEAL					
	FFA Basal (mmol/l)	Rise (mmol)	Glycerol Basal (mmol/l)	Rise (mmol)	Glucose Basal (mmol/l)	Rise (mmol)	Insulin Basal (m units/l)	Change (m units)	Glucose Basal (mmol/l)	Rise (mmol)	Insulin Basal (m units/l)	Rise (m units)
Metformin	0.506 ±0.066	21.01 3.45	0.121 ±0.014	3.419 0.740	9.0 ±0.85	51.40* ±7.70	26.00 ± 7.18	-251.2 ± 94.8	8.92 ±0.885	306.9 ± 50.5	27.40 ± 7.95	4478 ±1368
Sulphonylurea	0.502 ±0.092	21.1 3.96	0.138 ±0.170	3.530 0.864	8.65 ±0.77	38.90 ± 7.89	30.60 ± 8.71	-349.3 ± 146.8	8.96 ±0.79	267.20 ± 42.24	29.70 ± 8.39	6136* ±1770

Table 4.2: Basal and accumulative change of biochemical indices during noradrenaline infusion and a meal.

Comparison of metformin therapy vs sulphonylurea therapy      \*p<0.05.  
Mean ± SEM.

#### 4.3.2. Body Weight

There was no significant change in body weight after 2 weeks on alternative therapy (Table 4.3).

#### 4.3.3. Resting Metabolic Rate

There was no significant change in RMR on metformin or sulphonylurea therapy (Table 4.3) and this was statistically similar and closely correlated ( $r = 0.96$ ;  $p < 0.0001$ ) with the predicted value based on age, sex and surface area ( $5.20 \pm 0.26$  kJ/min).

#### 4.3.4. Thermic Response to the Meal

The rise in metabolic rate in response to the mixed meal following the administration of either metformin or sulphonylurea is illustrated in Figure 4.2(a) and was similar on both drugs. There was no significant difference in the accumulative thermic response to the meal over 2 hours on either therapy (Table 4.3). The mean difference of +1.65 KJ had a  $t$  value of 0.506 and 95% confidence limits of -6.4 to +9.7 KJ.

#### 4.3.5. Biochemical Response to the Meal

##### 4.3.5.1. Glucose

The mean fasting plasma glucose prior to meal ingestion was similar on metformin and sulphonylurea therapy. Although the mean plasma glucose at 30, 60, 90 and 120 minutes following the meal was greater on metformin this did not reach statistical significance (Fig. 4.3). However, when the accumulative incremental rise of glucose over the 2 hour period was calculated, this was significantly greater on metformin compared to sulphonylurea (Table 4.2).

	Weight (kg)	RMR (kJ/min)	Thermic Response to Noradrenaline Infusion (kJ)	Thermic Response to Meal (kJ)
Metformin	89.2 ± 7.5	5.29 ± .041	23.11 ± 3.0	86.8 ± 10.8
Sulphonylurea	89.8 ± 7.7	5.34 ± 0.34	21.46 ± 1.87	75.8 ± 7.5

Table 4.3: Resting metabolic rate and accumulative incremental thermic response to noradrenaline infusion and meal.

Mean ± SEM. There was no statistical significance between the two groups.



Fig. 4.2(a)

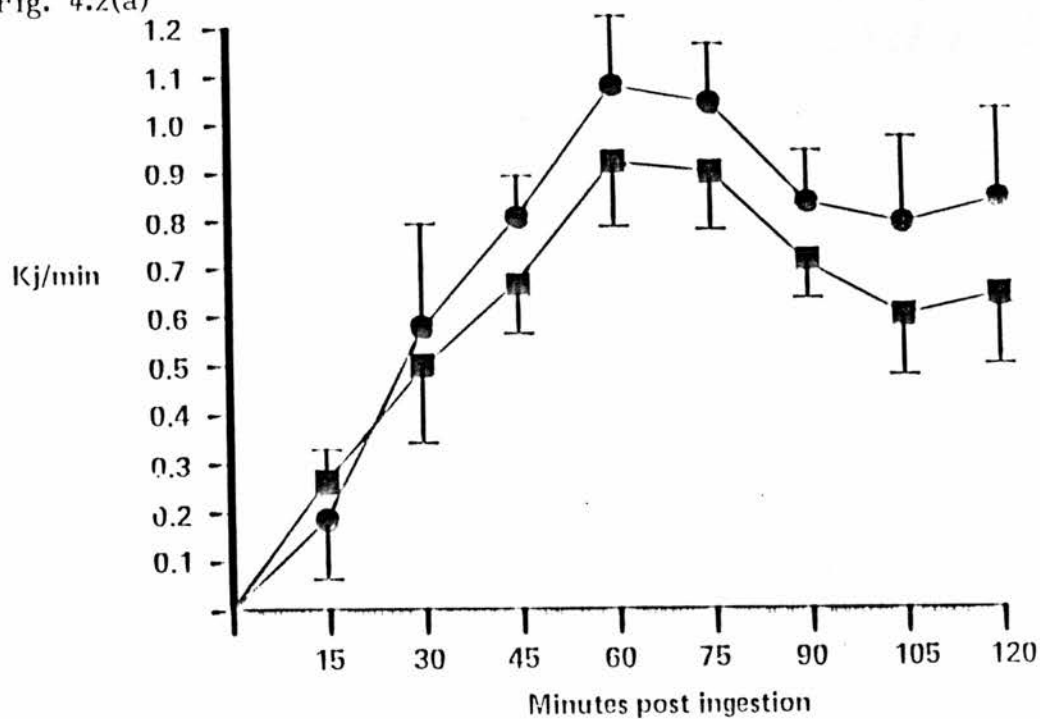


Fig. 4.2(b)

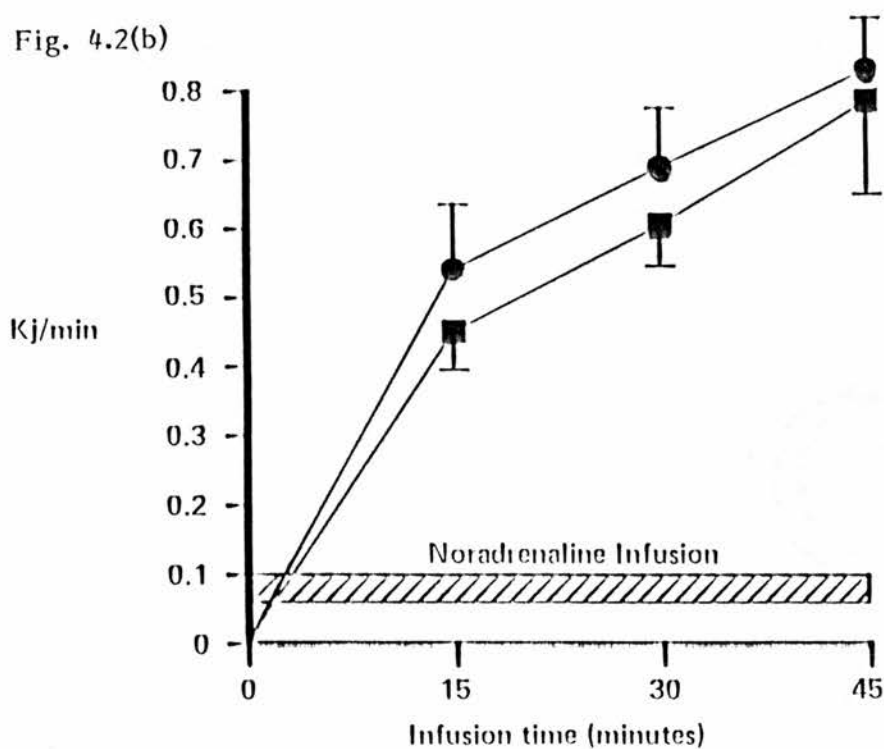


Fig. 4.2 Rise in metabolic rate following meal ingestion and during noradrenaline infusion on metformin (●) and sulphonylurea (■) therapy

(a) Meal ingestion

(b) Noradrenaline infusion. There were no significant differences between groups. Mean  $\pm$  SEM.

Fig. 4.3(a) PLASMA GLUCOSE RESPONSE TO MEAL

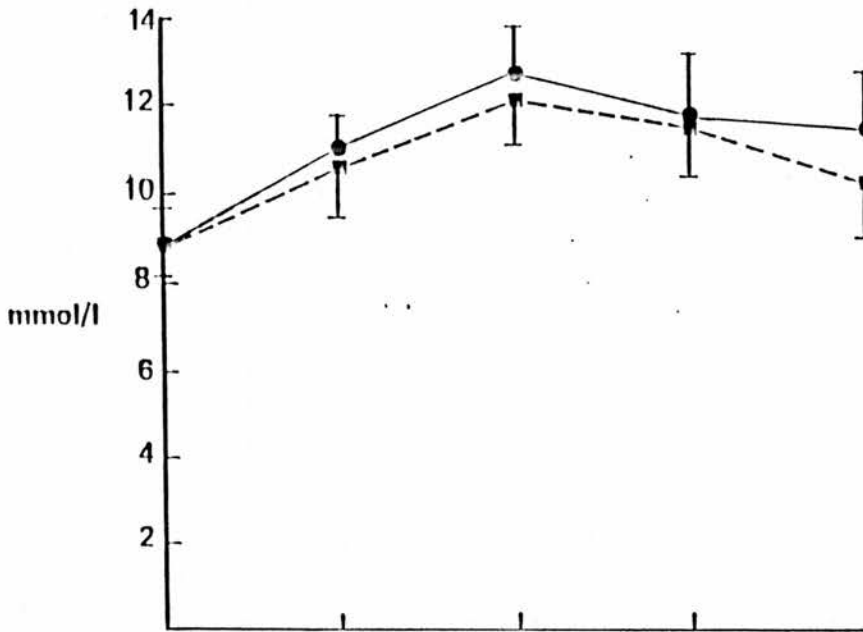


Fig. 4.3(b) PLASMA INSULIN RESPONSE TO MEAL

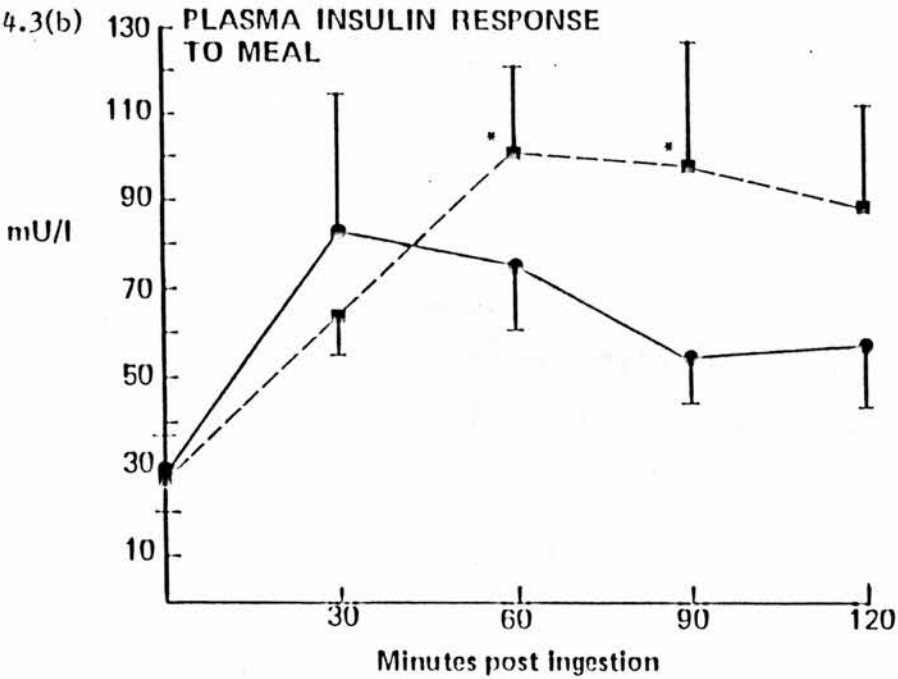


Fig. 4.3 Plasma glucose and insulin response to meal on sulphonylurea (■) and metformin (●) therapy

- (a) Plasma glucose response  
(b) Plasma insulin response

\* $p > .05$ .

#### 4.3.5.2. Insulin

Plasma insulin concentrations were significantly greater at 60 and 90 minutes (Fig. 4.3) following meal ingestion as was the accumulative incremental rise of insulin in response to the meal on sulphonylurea therapy (Table 4.2).

#### 4.3.6. Thermic Response to Noradrenaline

Noradrenaline infusion resulted in a significant rise in metabolic rate in all subjects and the mean absolute (Fig. 4.2(b)) and accumulative increment rises (Table 4.3) in response to the infusion were similar on both therapies. The mean difference of 11 KJ had a t value of 0.95 and 95% confidence limits -17 to +39 KJ.

#### 4.3.7. Biochemical Response to Infused Noradrenaline

##### 4.3.7.1. Glucose

Plasma glucose rose significantly on both therapies during noradrenaline infusion (Fig. 4.4(a)). The accumulative incremental rise of glucose (Table 4.2) was however significantly greater on metformin.

##### 4.3.7.2. Insulin

There was a biphasic response of plasma insulin to the continued effects of fasting and noradrenaline infusion. After 15 minutes there was a significant fall in plasma insulin on both metformin and sulphonylurea (Fig. 4.4(b)). During the subsequent 30 minutes plasma insulin concentrations increased to that approaching the fasting value. The accumulative net fall in plasma insulin was however similar on both therapies (Table 4.2).

Fig. 4.4(a)

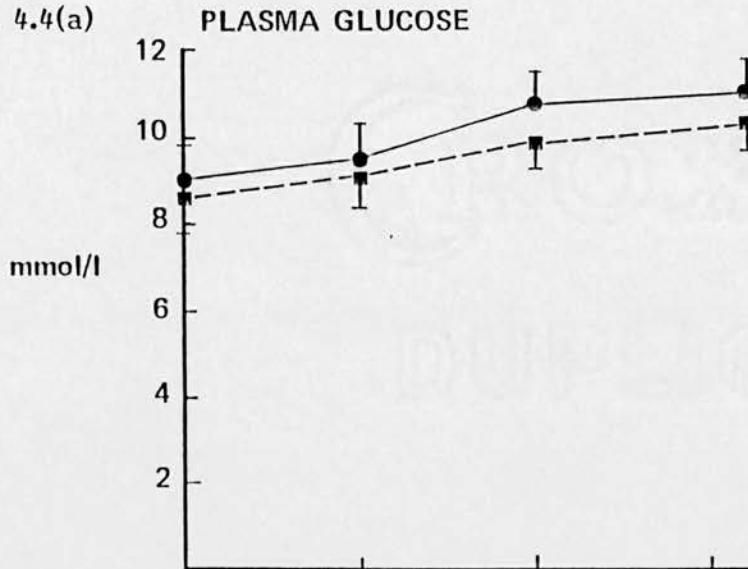


Fig. 4.4(b)

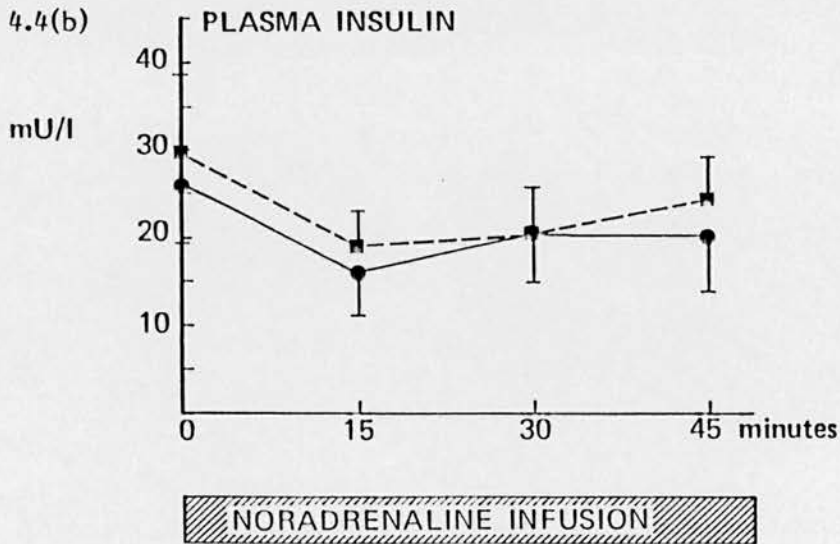


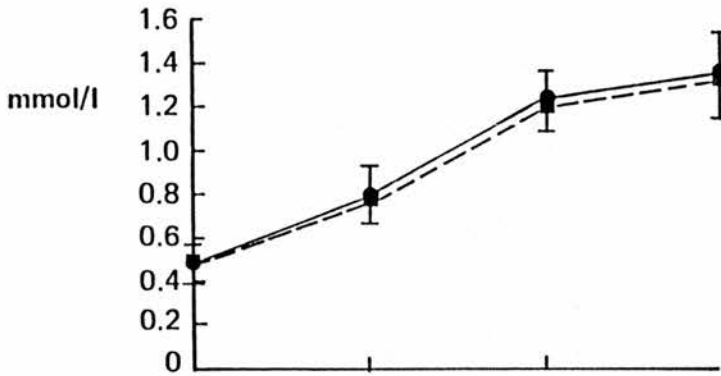
Fig. 4.4 Plasma glucose and insulin response to noradrenaline infusion on metformin (●) and sulphonylurea (■) therapy

- (a) Plasma glucose response  
(b) Plasma insulin response

There were no significant differences between groups.

4.5(a)

# PLASMA NEFA DURING NORADRENALINE INFUSION



4.5(b)

# PLASMA GLYCEROL

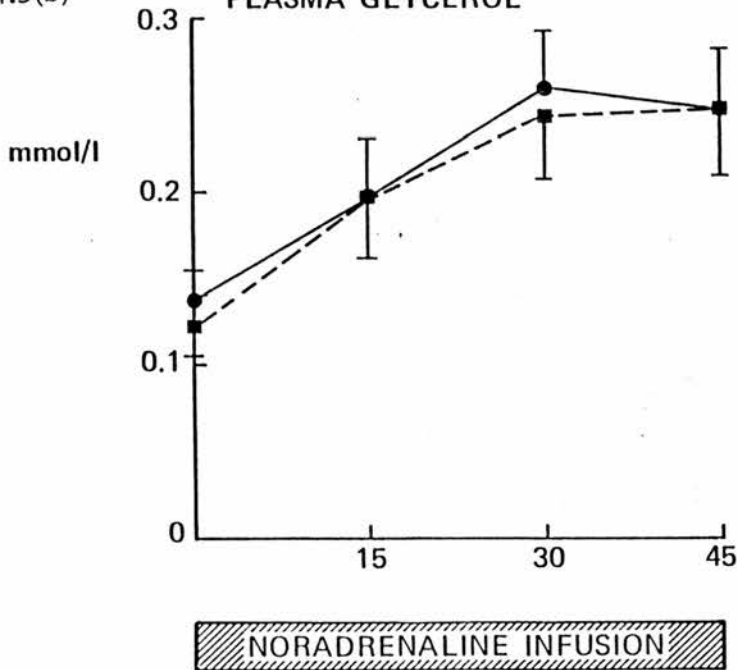


Fig 4.5 Lipolytic response to noradrenaline infusion.  
Metformin (●); Sulphonylurea (■).

4.5 (a) Plasma non-esterified fatty acids (NEFA) during noradrenaline infusion.

4.5 (b) Plasma glycerol during noradrenaline infusion.

There were no significant differences between groups.

#### 4.3.7.3. Glycerol and free fatty acids

Fasting plasma glycerol and free (non-esterified) fatty acid concentrations were similar on metformin and sulphonylurea treatment as were the lipolytic responses to infused noradrenaline (Fig. 4.5 and Table 4.2).

#### 4.4. DISCUSSION

The resting metabolic rate and the increases in heat production consequent to food and catecholamine stimulation can account for 80 to 90% of total daily energy expenditure. Measurement of these parameters in Type II diabetic subjects treated with metformin or a sulphonylurea under similar conditions of glycaemic control failed to demonstrate any alteration of energy expenditure on either therapy.

Thus it would appear that, at least in the short term, the weight loss observed during metformin therapy and conversely the weight gain during treatment with a sulphonylurea derivative (Clarke and Duncan, 1968) are not facilitated by major changes in energy output. It is unlikely that the lack of any consistent difference between metformin and sulphonylurea was due to cross interference with the previous therapy or to an inadequate period of therapy with the other. Metformin reaches a maximum plasma concentration within 2 hours of dosage and has a plasma half life of 4 to 8 hours (Tucker et. al., 1981). The sulphonylurea agents used in this study all reach peak plasma concentrations within 2 hours of dosage and as can be seen from Figure 4.4 result in significant insulin release after minutes. Chlorpropamide has the longest half life of the sulphonylurea agents at 35 hours (Martindale, 1982) but nevertheless there is no evidence to suggest that these drugs would remain in the tissues 14 days after cessation of therapy in concentrations

likely to interfere with the thermic responses on subsequent therapy.

One can argue, however, that as significant changes in body weight do not occur until after several months of therapy with either drug (Clarke and Duncan, 1968) changes in energy expenditure may not be demonstrable after 2 weeks of therapy. However, the study was deliberately designed to be of sufficient duration to ensure therapeutic effect and yet avoid significant alterations in body weight and fat free mass, as these changes per se would have resulted in alteration of the RMR. Thus, if metformin therapy had resulted in weight loss the resultant fall in the RMR secondary to the reduction in fat free mass would confound clear interpretation of an effect of metformin on energy expenditure.

By exclusion, therefore, alterations in available energy intake are most likely to account for the considerable net weight change of 6.6 kg over the 24 month period of study in Clarke and Duncan's original description (1968). These workers calculated that this would represent an average increase in daily energy intake in the region of 110 kcals. By comparison, it has been demonstrated in Chapter III that alterations of energy expenditure in Type I diabetic subjects of a similar magnitude (89 kcals/day) can also result in a significant increase in weight (Section 3.3.2).

Assessment of daily energy intake was not carried out during this study but it is interesting to note that even in this small group of subjects one third of those subjects noted mild to moderate gastrointestinal symptoms during metformin therapy which could result in the reduction of appetite or absorption of nutrients. These symptoms, however, tended to be transient and Clarke and Duncan (1968), who described a similar incidence of side effects, failed to find a relationship between those patients who achieved weight loss and those patients who had symptoms of anorexia.

To detect a caloric deficit in the region of 100 kcal/day would require a consistent accuracy of recording of at least 90-94% of the daily energy input. In the free-living subject, studied outwith the confines of a metabolic ward, even weighed daily diary recording of intake is unlikely to achieve an accuracy of this degree and even if it approaches this, the methods involved will result in significant alteration of eating behaviour which will negate the object of the study (Garrow, 1978).

It has also been suggested, mainly from animal studies (see Section 4.1) that metformin may reduce available intake of the major nutrients via diminished intestinal absorption (Section 4.1). It is not possible to comment on the absorption of nutrients from the present study. The significantly greater rise of plasma glucose following meal ingestion on metformin therapy would not suggest inhibition of intestinal absorption of glucose but the concurrent attenuation of the rise of plasma insulin compared to that on sulphonylurea is probably the major factor influencing this difference.

The exaggerated insulin response to a meal and glucose on sulphonylurea is well recognised (Abramson and Arky, 1967) and may further tend to promote weight gain through increased glucose utilisation and lipogenesis in the Type II diabetic subject on sulphonylurea.

The individual components of energy expenditure in the treated Type II diabetic subject will now be considered with reference to the suggestion that impaired glucose tolerance may act to predispose the diabetic subject to weight gain due to blunted energy expenditure (Felig, 1984).

Up to 70% of total daily energy expenditure can be accounted for by the RMR and therefore this component acts as one of the major determinants of energy output in man (Garrow, 1985).



The RMR of the Type II diabetic subjects studied was not significantly different from that predicted for non-diabetic subjects based on age, sex and surface area. Therefore this component does not act to limit energy expenditure in the Type II diabetic subject. Conversely, the total 24 h energy expenditure of an overweight individual will in fact be greater than that of a sex and age matched lean individual due to the mathematical relationship of the RMR to surface area (Boothby and Sandiford, 1929) or fat free mass (Ravussin et. al., 1982) and the additional expenditure involved in movement of the increased body mass.

The normal RMR of the Type II diabetic subject is in contrast to that previously described in the poorly controlled diabetic Type I diabetic subject (Section 3.3.4). Although elevation of the plasma glucose appeared to be a major determinant of this abnormality in Type I diabetic subjects (Section 3.3.4) it is not possible to comment whether the normal RMR in the Type II diabetics reflects the better degree of glycaemic control or a further fundamental difference between Type I and Type II diabetes mellitus. Recent data from Nair et. al. (1986) has addressed this problem and contrasts with the present experience in that not only did the obese type II diabetic subjects demonstrate a significantly raised RMR compared to predicted but also those subjects with impaired glucose tolerance who were not frankly diabetic. These differences may be partly accounted for by the gross obesity of the subjects studied ( $\text{BMI } 38.2 \pm 3.3 \text{ kg/m}^2$ ) and the "discontinuation of therapy for a period of one week" prior to study but it is still surprising that those subjects with glucose intolerance (mean 2 hour 75 g glucose tolerance test glucose concentration of 9 mmol/l) had a significantly raised RMR if glycaemic control is one of the major factors in determining the increase in RMR in diabetes mellitus (Section 3.3.3).

As differences in the RMR between lean and obese subjects does not reveal any evidence of decreased energy output in the obese state attention has been focussed on dynamic aspects of energy expenditure such as the responses to food and catecholamines.

The thermic response to the mixed constituent meal was unaltered in the Type II diabetic subject whether on metformin or sulphonylurea therapy and further, was similar to that observed in younger lean non-diabetic subjects ( $72.2 \pm 8.4$  kJ/min; Table 3.6) and Type I diabetic subjects on conventional therapy ( $73.6 \pm 12.3$  kJ/min; Table 3.6) and CSII ( $71.0 \pm 12.1$  kJ/min; Table 3.6).

This is in marked contrast to the reports of blunted thermic responses to infused (Ravussin et. al., 1983) and oral (Golay et. al., 1982; Schutz et. al., 1984b) glucose in obese Type II diabetics. The latter workers further hypothesised that as this abnormality persisted despite weight loss it may act as a factor predisposing to the relapse of the obese state. The composition of the more physiological meal used in the present study compared to large doses of infused and oral glucose has already been discussed in Section 3.4.3 and may partly explain the conflicting evidence but two other factors may be of importance. Firstly the Type II subjects recruited for the previous studies were selected not only on the basis of Type II diabetes mellitus but also had a family history of obesity and were grossly obese with a %Ideal Body Weight in excess of 150%. Secondly, these subjects were not receiving a carbohydrate restricted diet (maintenance diet contained 250 to 300 g carbohydrate daily) and had not received oral medication for at least 48 hours prior to study.

Although, therefore, it is not possible to cross compare these studies with the present work it can be concluded that in Type II diabetic subjects controlled with diet and oral hypoglycaemic agents, who were not selected

on the basis of obesity, do not demonstrate an abnormality in the thermic response to a mixed constituent liquid meal.

Finally, to a consideration of the thermic response to catecholamine infusion in Type II diabetes. The thermic and lipolytic responses to noradrenaline were also similar on metformin and sulphonylurea and to those reported for younger lean, non diabetic subjects ( $27.1 \pm 3.4$  kJ/min; Table 3.6) and those recalculated from Finer et. al.(1985) (21.9 kJ/min) using a similar noradrenaline infusion regimen.

This contrasts with the persistent blunting of the thermic responses to infused noradrenaline in Type I diabetic subjects irrespective of the degree of glycaemic control (Table 3.6) and obese subjects of similar age to the current study before and after weight loss (Jung et. al., 1979). As these abnormalities have also been demonstrated in the non-obese lactating subject with apparent increased insulin sensitivity (Illingworth et. al., 1986) it would appear that abnormal glucose tolerance and the obese state are not prerequisites of this abnormality. This suggests that some other factor or factors unrelated to insulin responsiveness, and as yet unidentified, modulates the physiological thermic response to infused catecholamines.

Chapter V

The Contribution of Brown Adipose Tissue to  
Thermogenesis in Man

## SUMMARY

In order to assess the histological, biochemical and functional characteristics of brown adipose tissue in adult man, perirenal fat was obtained from 31 subjects either at post mortem or during renal surgery.

Brown adipose tissue was demonstrable macroscopically in 16 out of the 20 subjects (80%) in whom the total perinephric fat depot was obtained. Histological studies confirmed the presence of multilocular adipocytes with dense mitochondria associated with a rich vascular supply and sympathetic innervation.

Measurement of GDP binding demonstrated the presence of uncoupling protein, the prerequisite for brown adipose tissue thermogenesis, in concentrations intermediate to that of the warm and cold adapted guinea pig. Function of the uncoupling protein was demonstrated by fatty acid stimulation and GDP inhibition of respiration in isolated mitochondria and these data indicate some potential for thermogenesis in brown adipose tissue in adult man.

However, calculation of the contribution of this tissue to the in vivo response to noradrenaline, extrapolated from data of the tissue cytochrome c oxidase activity and the in vitro response of isolated brown adipocytes to noradrenaline, suggests that this tissue only accounts for 0.2% of the thermic response to infused noradrenaline.

Thus although brown fat has been found to be quantitatively important in animal studies, considerable caution must be exercised in extrapolating its significance to adult man.

### 5.1. INTRODUCTION

As reviewed in Chapter I, brown fat thermogenesis in small mammals adapts to both temperature (Himms-Hagen, 1984) and dietary stimuli (Rothwell and Stock, 1979) to produce heat for the maintenance of body temperature in the former situation and to dissipate excess calories in the latter.

In adult man there is evidence to support similar adaptive changes in non-shivering thermogenesis (NST) occurring in response to cold (Joy, 1963) and overfeeding (Sims, 1976). Presumptively, by analogy with animal models and morphological extrapolation brown adipose tissue has been implicated as the effector of NST although no direct role has been conclusively demonstrated. Accordingly, defective brown adipose tissue thermogenesis in obese adult man similar to that observed in some genetically obese rodents has been speculated (Jung et. al., 1979).

However, as outlined in the preceding chapters, despite extensive research on many aspects of energy balance the cumulative energetic significance of blunted thermic responses in obese (Jung et. al., 1979; Shetty et. al., 1981) Type I (Leslie et. al., 1986) and Type II (Golay et. al., 1982) diabetic and lactating subjects (Illingworth et. al., 1986) remains a subject of controversy. A major obstacle to the interpretation of these data obtained during various physiological and pathological states is the lack of understanding of the basic physiological mechanism or mechanisms involved in the generation of these thermic responses in man.

The present study was therefore designed to firstly, identify histologically and biochemically the presence of functional brown adipose tissue in the adult. Secondly, to attempt to quantify the proportion of the thermic response to infused noradrenaline that can be accounted for by heat production from this tissue. Finally, from this data it was therefore hoped to

be able to comment on whether abnormalities of this tissue can be implicated in the blunted responses to infused noradrenaline discussed in Chapter III.

## 5.2. EXPERIMENTAL PROCEDURE

### 5.2.1. Tissue Samples

As discussed in Chapter I, perirenal fat was chosen as the site for study as this is one of the major sites of brown adipose tissue in adult man (Table 1.1) and is also accessible for study both at post mortem and in the living subject during surgical procedures involving the kidney. None of the subjects from whom tissue was obtained were obese (BMI less than 28.6) nor known to suffer from diabetes mellitus.

#### 5.2.1.1. Post-mortem tissue (samples 1-10; Table 5.1)

Post-mortem tissue was obtained from cadavers, less than 18 h after death who had died suddenly, by dissection of the total perinephric fat from one kidney. The specimens were transported to the laboratory in an ice cold solution containing 250 mmol/l sucrose, 10 mmol/l TES (2 [2-hydroxy-1, 1-bis(hydroxymethyl)ethyl]-aminoethane sulphonic acid), 1 mmol/l EDTA at pH 7.2.

Macroscopic examination of the major fat depots for the presence of brown areas was performed in the post mortem subjects (subjects nos. 2, 3 and 4). Abdominal wall, anterior and posterior thigh, interscapular and omental adipose tissue and the fat depots associated with the abdominal and thoracic aorta, internal and external carotid arteries as well as the pericardial and perirenal fat deposits were examined.

Table 5.1 (overleaf)



(c) Nephrectomy

22	1/52/M	Hydronephrosis	4	-	Detected
23	11/F	Malignant hypertension	12	2.9	24
24	15/F	Nephroblastoma	10	1.0	10
25	18/M	Hydronephrosis	15	1.05	7
**26	19/F	Subarachnoid haemorrhage	71	12.1	17
**27	25/M	Subarachnoid haemorrhage	60	0.84	1.4
28	34/F	Hydronephrosis	32	1.6	5
29	39/M	Hydronephrosis	85	5.9	7
30	51/F	Hypernephroma	49	24	4.9
31	69/M	Hypernephroma	70	-	Detected

Table 5.1: Subject details from whom perirenal fat obtained.

- (a) Post mortem tissue (Nos. 1-10)  
(b) Biopsy samples (Nos. 11-21)

\*denotes periadrenal fat obtained at removal of pheochromocytoma (17,18) or bilateral adrenalectomy (20).

- (c) Nephrectomy (Nos. 22-31)

\*\*denotes perirenal fat obtained at renal transplant donation.

"Detected" refers to those samples in which areas of brownish discolouration were apparent but due to small volume and distribution separate dissection was not possible.

#### 5.2.1.2. Per-operative tissue (Table 5.1)

Tissue was obtained during surgical procedures involving the kidney either during nephrectomy when the total perinephric fat was obtained (subject nos. 22-31; Table 5.1) or during procedures when the retroperitoneal area was exposed (subject nos. 11-21; Table 5.1). These samples consisted of small biopsies (1-4 g net weight) selected by the surgeon on the basis of ease of attainment.

Tissue for mitochondrial preparation was transported to the laboratory in a similar medium to that for post mortem tissue (Section 5.2.1.1.). When isolated adipocytes were to be prepared, this was substituted for by "cell isolation medium", containing 110 mmol/l NaCl, 5 mmol/l KCl, 5 mmol/l  $\text{NaH}_2\text{PO}_4$ , 1.5 mmol/l  $\text{KH}_2\text{PO}_4$ , 5 mmol/l  $\text{NaHCO}_3$ , 10 mmol/l pyruvate, 10 mmol/l TES, 1.4 mmol/l  $\text{Mg SO}_4$ , 1.5 mmol/l  $\text{CaCl}_2$ , 10 mmol/l glucose, 10 mmol/l fructose, 64  $\mu\text{mol/l}$  albumin at pH 7.4 and 37°C.

### 5.2.2. Anatomy

#### 5.2.2.1. Macroscopic appearance

Tissue was examined macroscopically in theatre prior to transport for the presence of brown discolouration which is typical of thermoregulatory active brown adipose tissue in animals, and samples taken for histological examination.

#### 5.2.2.2. Light microscopy

Material for light microscopy was fixed in 10% formol saline prior to processing as follows: 1) paraffin embedding and sectioning at 10  $\mu\text{m}$  for haematoxylin and eosin (H and E) staining or for silver impregnation by the Holmes method and 2) freeze microtomy at 15  $\mu\text{m}$  for the application of oil Red O method for lipid demonstration.

Material for fluorescence microscopy was frozen by dry ice onto chucks and transported by train to Professor J.D. Lever, University College, Cardiff for the demonstration of catecholamines. On receipt, cryostat sections 12-15  $\mu\text{m}$  thick were cut at  $-30^{\circ}\text{C}$ , thawed directly onto glass slides and processed by a modification of the sucrose-potassium phosphate-glyoxylic acid (SPG) technique of de la Torre and Surgeon (1976).

#### 5.2.2.3. Electron microscopy

Material for electron microscopy was fixed in ice-cold cacodylate-buffered 3% glutaldehyde for 4 hours prior to an overnight buffer wash and subsequent post fixation in 1% buffered osmium tetroxide before processing to araldite. Fine sections (600-900  $\text{\AA}$  thick) were stained with uranyl acetate and lead citrate prior to examination by Professor J.D. Lever (Cardiff) and Dr D. Hopwood (Dundee).

#### 5.2.3. Tissue Preparation

Tissue for mitochondrial preparation and adipocyte isolation was rapidly dissected free of blood vessels and connective tissue with fine scissors. In the majority of samples dark coloured foci were distinguishable amongst white coloured fat, weighed and processed separately. In those cases where delineation between light and dark areas was not possible the undifferentiated sample was pooled for subsequent analysis. Cleaned tissue was then minced finely with scissors.

##### 5.2.3.1. Mitochondrial preparation

Tissue for mitochondrial preparation was homogenised in six times its volume of 250 mmol/l sucrose, 10 mmol/l TES (sodium salt), 1 mmol EDTA at pH 7.2. The homogenate was subsequently filtered through 200  $\mu\text{M}$  nylon

mesh gauze and centrifuged at 12,000 r.p.m. for ten minutes. The supernatant was discarded and the pellet suspended in a medium consisting of 250 mmol/l sucrose, 120 mmol/l TES (sodium salt), 1 mmol/ EDTA and 64  $\mu$ mol/l bovine serum albumin at pH 7.2. This medium was used for all subsequent steps until the final spin. A low spin speed at 3,500 r.p.m. was conducted for ten minutes in order to pellet red blood cells. The supernatant was then centrifuged twice at 8500 r.p.m. for ten minutes. The final spin and resuspension was made in 100 mmol/l KCL, 5 mmol/l TES at pH 7.0.

#### 5.2.3.2. Adipocyte preparation

The minced tissue was pre-equilibrated at 37°C with 95% O<sub>2</sub> and 5% CO<sub>2</sub> in cell isolation medium (Section 5.2.1.2.) and incubated in a water bath for 15 minutes. Collagenase (2 mg/ml) and Ca<sup>++</sup> ions (1.5 mmol/l) were then added for 45 minutes. The tissue was then filtered through 200  $\mu$ M mesh nylon sieve. The cells were then suspended in cell isolation medium and washed twice at 300 g max for 3 minutes. After the final wash cells were counted in an improved Neubauer haemocytometer.

#### 5.2.4. Protein Determination

Mitochondrial protein was determined by the biuret method using bovine serum albumin as the standard.

#### 5.2.5. Cytochrome c Oxidase Activity

Cytochrome c oxidase is the terminal enzyme of the respiratory chain and therefore provides an index of mitochondrial density. Assay of the enzyme in brown adipose tissue immediately, and 48 h after death in guinea pigs demonstrated stability of the enzyme in post mortem material (D.G. Nicholls, personal communication). Huttunen et. al. (1981) have also

confirmed the stability of this enzyme up to 5 days post mortem in human pericardial fat.

50 to 100 mg of dark, light or undifferentiated tissue was homogenised in a phosphate buffered medium containing 0.5% lubrol and the enzymic activity determined polarographically at 37°C according to the method of Rafael (1983). Calculated values were expressed as  $\mu\text{moles O/min/g}$  tissue from homogenised tissue samples and as  $\mu\text{moles O/min/mg}$  mitochondrial preparations from light and dark areas.

From specimens in which the total perinephric fat depot was obtained the total capacity of the enzyme in the fat surrounding the kidney was calculated from the specific activity of each area and the percentage of light and dark areas in the total weighed sample. Biopsy samples were not quantifiable in terms of total perirenal capacity.

#### **5.2.6. Mitochondrial Respiratory and Membrane Potential Measurements**

A thermostatically controlled perspex chamber was fitted with a Clarke oxygen electrode and tetraphenyl phosphonium (TPP) sensitive electrode with remote KCL reference, allowed a simultaneous monitoring of respiration and membrane potential (Locke et. al., 1982). Mitochondria (0.3 mg/ml) were incubated in a medium containing 50 mmol/l KCL, 10 mmol/l TES, 2 mmol/l EGTA, 5 mmol/l phosphate (sodium salt), 10 mmol/l pyruvate, 6 mmol/l malate, 10 mmol/l sn-glycerol-3-phosphate, 2  $\mu\text{g/ml}$  oligomycin, 5  $\mu\text{mol/l}$  TPP and 16  $\mu\text{mol/l}$  albumin at pH 7.0, 30°C.

To demonstrate respiratory control successive additions of 64  $\mu\text{mol/l}$  bovine serum albumin, 3 mmol/l GDP and 2 aliquots of palmitate to create a final fatty acid to albumin molar ratio of 1:1 and 2:1 were made (Fig. 5.6).

### 5.2.7. Mitochondrial GDP Binding

The mitochondrial complement of uncoupling protein can be determined by a purine nucleotide binding assay. The tritiated analogue of guanosine 5' diphosphate (GDP) was employed as it has high affinity for the nucleotide binding site of 32D uncoupling protein and its exclusion from the mitochondrial matrix (Nicholls, 1976).

Mitochondria (0.3 mg/ml) were incubated at 30°C pH 7.0 in 700 µl of nucleotide binding medium containing 100 mmol/l acetate (potassium salt), 5 mmol/l TES, 16 µmol/l albumin, 1.3 µmol/l rotenone, 10 µmol/l (<sup>3</sup>H) GDP (10 µCi/ml) and 0.3 µCi/ml (<sup>14</sup>C)-sucrose. Binding was determined at a single 10 µmol/l GDP concentration (Rial et. al., 1983).

### 5.2.8. Calculation of estimated in vivo contribution of brown adipose tissue to noradrenaline stimulation.

#### 5.2.8.1. In vitro response to noradrenaline

Ideally, in order to estimate the contribution that brown adipose tissue could make to noradrenaline stimulated thermogenesis, isolated adipocytes should be prepared in vitro under conditions equivalent to in vivo responsiveness. The increase in respiration of these cells on addition of noradrenaline could then be expressed as a percentage of the mitochondrial density (cytochrome c oxidase activity). From a knowledge of total cytochrome c oxidase activity of the tissue, the in vivo response of that tissue can be extrapolated. Isolated brown adipocytes from human subjects were prepared as described (Section 5.2.3.2.) in parallel with those from the dorsal fat pad of warm adapted and cold adapted guinea pigs of either sex maintained at either 28-31°C (warm adapted) or 4-7°C (cold adapted) for at least 2

weeks. Basal and stimulated respiration were measured in the oxygen electrode. Maximal stimulation was achieved with the addition of noradrenaline to a final concentration of 10  $\mu\text{mol/l}$ . A sample calculation and the assumptions made in this calculation are discussed in the results section (5.3.7.1).

#### 5.2.8.2. In vivo response to noradrenaline

The eight non-diabetic subjects described in Chapter III (Section 3.2.1.2) who were infused with 0.1  $\mu\text{g/kg IBW/min}$  of noradrenaline for 45 minutes (Section 2.1.5.2) were used to assess this parameter. The maximal in vivo response was taken as the difference between the mean basal oxygen consumption and that obtained during the final 15 minutes of the noradrenaline infusion and expressed in  $\text{ml O}_2/\text{min}$ .

### 5.3. RESULTS

#### 5.3.1. Anatomy

Macroscopic examination of the major fat depots for the presence of brown adipose tissue was performed on three post-mortem subjects (Nos. 2, 3, 4). Apart from the perirenal area in all subjects and a small deposit around the abdominal aorta at the origin of the renal arteries in subject no. 2, macroscopic and histological brown adipose tissue was not demonstrable. Those areas sampled consisted of unilocular cells and in all cases apart from the perirenal site, it was not possible to prepare a sufficient mitochondrial pellet from less than 6 g of tissue indicative of a low mitochondrial density and metabolic activity.

Thus from this small pilot study, which confirmed the data of Heaton et. al. (1972) subsequent attention was focussed on perirenal adipose tissue.

#### 5.3.1.1. Macroscopic Appearance (Table 5.1, Fig. 5.1)

Macroscopically areas of brown colouration were demonstrated in 16 out of the 20 samples (80%) when the total perinephric fat depot (post mortem and nephrectomy tissue) was obtained. This could be formally dissected, quantified and studied separately in 13 of these samples.

Biopsy samples usually weighing less than 4 g yielded a similar percentage of positive identifications (8 out of 11 samples) but was more difficult to quantify formally (5 out of 11 samples).

The distribution of the brown fat varied from well demarcated areas (Fig. 5.1) to small "islets" less than 5 mm in diameter distributed uniformly through the fat which made separate dissection impossible (6 out of 31 samples).

Deposits could be found with equal occurrence at the anterior, posterior, inferior and superior poles of the kidney as well as the hilum and periadrenal area.

All samples received from subjects under the age of 50 years where the total perinephric fat was obtained displayed localised areas of distinct brown colouration. Three positive identifications (one in a patient aged 69 years) out of six were recorded from older patients.

The net weight of dissected brown fat varied from detectable to 24 g. This latter specimen was obtained from a 51 year old subject who had a large hypernephroma with pericapsular extension of tumour. Histological examination of the brown discoloured areas revealed these contained multilocular adipocytes and not tumour cells.





10 cm.

Fig. 5.1. Macroscopic appearance of perinephric fat from subject no. 5 (Table 5.1) obtained at post mortem. Clear demarcation is seen between the brown adipose tissue (upper arrow) and white adipose tissue (lower arrow).

### 5.3.1.2. Phaeochromocytoma (Subjects 17 and 18)

In the two subjects diagnosed with phaeochromocytoma both had a greater than 2 year history of poorly controlled hypertension.

Subject No. 17 had raised urinary metanephrine excretion on three 24 hour urine specimens and a resting supine plasma noradrenaline concentration of 31.2 nmol/l (normal resting supine range up to 4 nmol/l) and resting supine adrenaline concentration of 5.2 nmol/l (reference range up 1.5 nmol/l). Subject No. 18 had a peripheral venous plasma noradrenaline concentration of 350 nmol/l and adrenal venous blood obtained peri-operatively had a concentration of 794 nmol/l. In neither subject was a brown fat tissue mass or hibernoma (Section 1.5.2.1) visualised at operation. Periadrenal fat demonstrated scattered foci of brown tissue in subject no. 18 and was undetectable in subject no. 17.

### 5.3.1.3. Light Microscopy

Thermogenically active brown adipocytes in the small mammal can be identified under the light microscope by the presence of granular multilocular adipocytes. The typical appearance of multilocular brown adipocytes adjacent to unilocular adipocytes obtained from subject no. 4 is shown in Fig. 5.2. The areas of multilocularity are associated with a rich vascular capillary supply whereas those areas where unilocular cells predominate appear to have a sparse vascular supply. These unilocular adipocytes are typical of white adipocytes but equally well may represent quiescent brown fat cells as suggested by the presence of GDP binding (uncoupling protein) in macroscopically light areas. After silver impregnation an abundant vasomotor nerve plexus was discernible on the walls of small arteries and nerves were traced in parenchymal distribution between multilocular adipocytes. Within these plexuses were putative

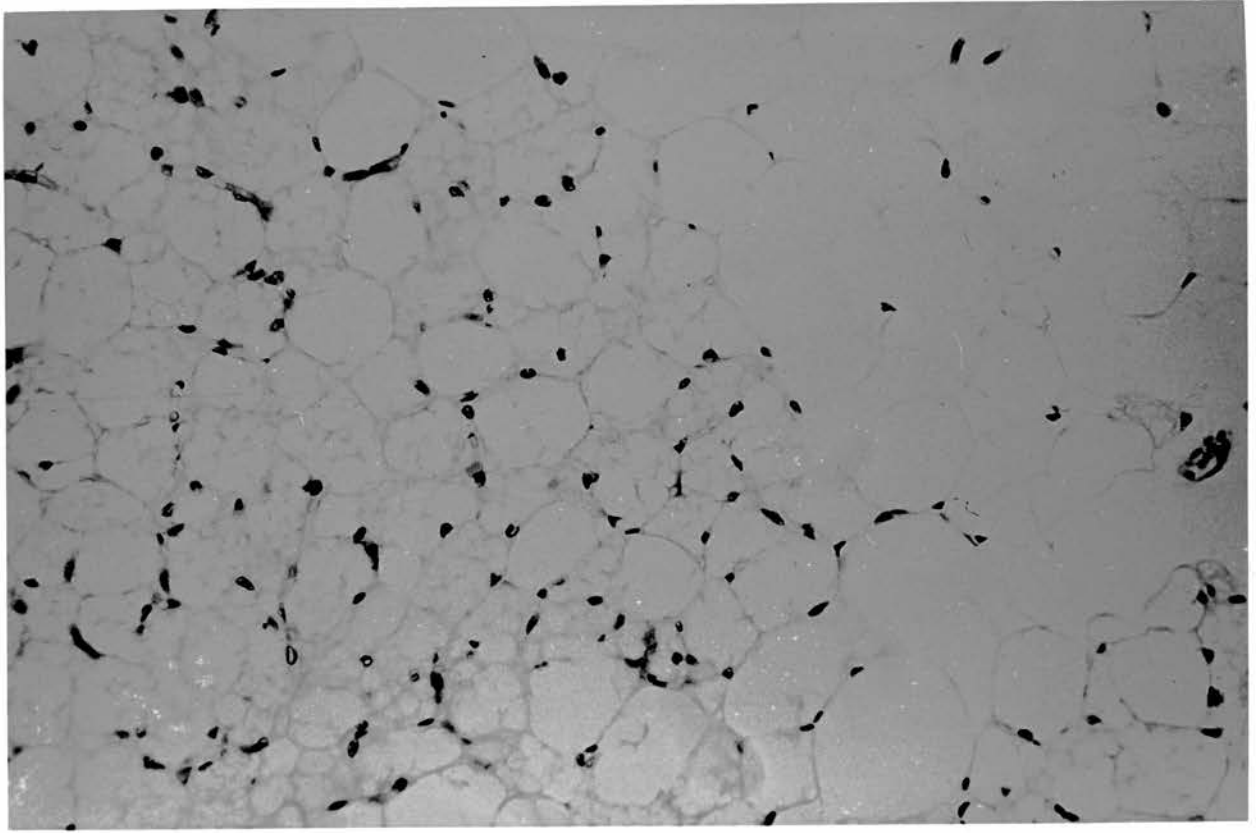


Fig. 5.2 Paraffin section of perinephric adipose tissue obtained from subject no. 4 stained with haematoxylin and eosin X 920. To the left of the photograph is seen the typical multilocular appearance of brown adipocytes in contrast to the unilocular white adipocytes on the right.

catecholaminergic nerve fibres characterised by fluorescence in SPG preparations (Fig. 5.3).

#### 5.3.1.4. Electron Microscopy

Fig. 5.4 shows an electron micrograph representative of perirenal 'dark' tissue. The large spherical nucleus, with prominent nucleoli, the multilocular fat droplets and numerous mitochondria with involuted dense cristae are demonstrated.

Fig. 5.5 shows an unmyelinated axon bundle in an intercellular space between brown adipocytes. Terminal features were observed in many of these nerves and included the presence of 800-1000 Å diameter dense cored vesicles as well as a number of 400-600 Å diameter clear vesicles.

#### 5.3.2. Cytochrome c Oxidase Activity

Cytochrome c oxidase activity was measured in the tissue homogenates and respective mitochondria in 12 subjects (Table 5.2) and was consistently less than comparable data obtained from warm and cold adapted guinea pigs. From these parameters the complement of mitochondrial protein per gramme of tissue (a more accurate estimate of mitochondrial density) can be estimated and this demonstrates two features.

Firstly, dark areas are more abundant in their complement of protein than are light areas indicating greater metabolic activity and, secondly, only in one subject (subject no. 23) did the protein complement approach that of the warm adapted guinea pig.

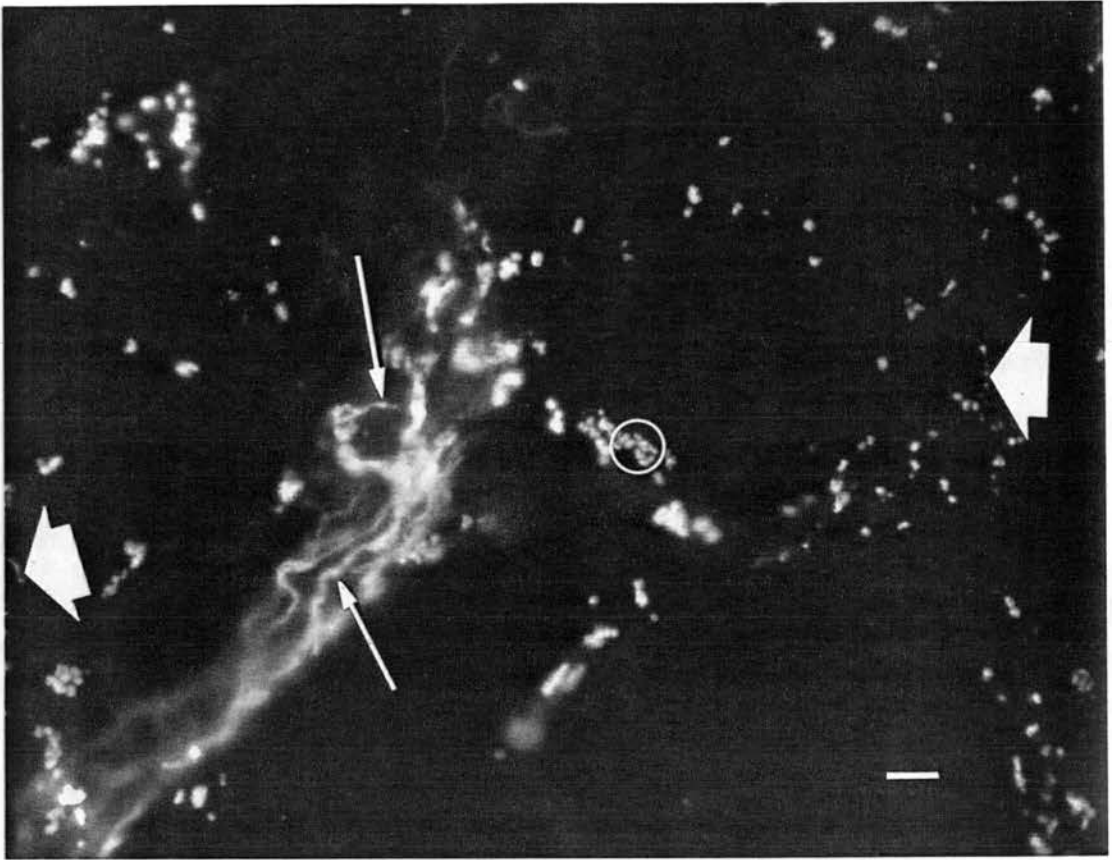


Fig. 5.3 Fluorescence micrograph of glyoxylic acid-treated human perirenal tissue from 39 year old subject. Scale base 10  $\mu\text{m}$  (X 750). Note beaded fluorescent axons on the walls of an artery (light arrows) and in parenchymal distribution (heavy arrows). Also note widespread distribution of lipofuchsin granules (circle) within adipocytes.

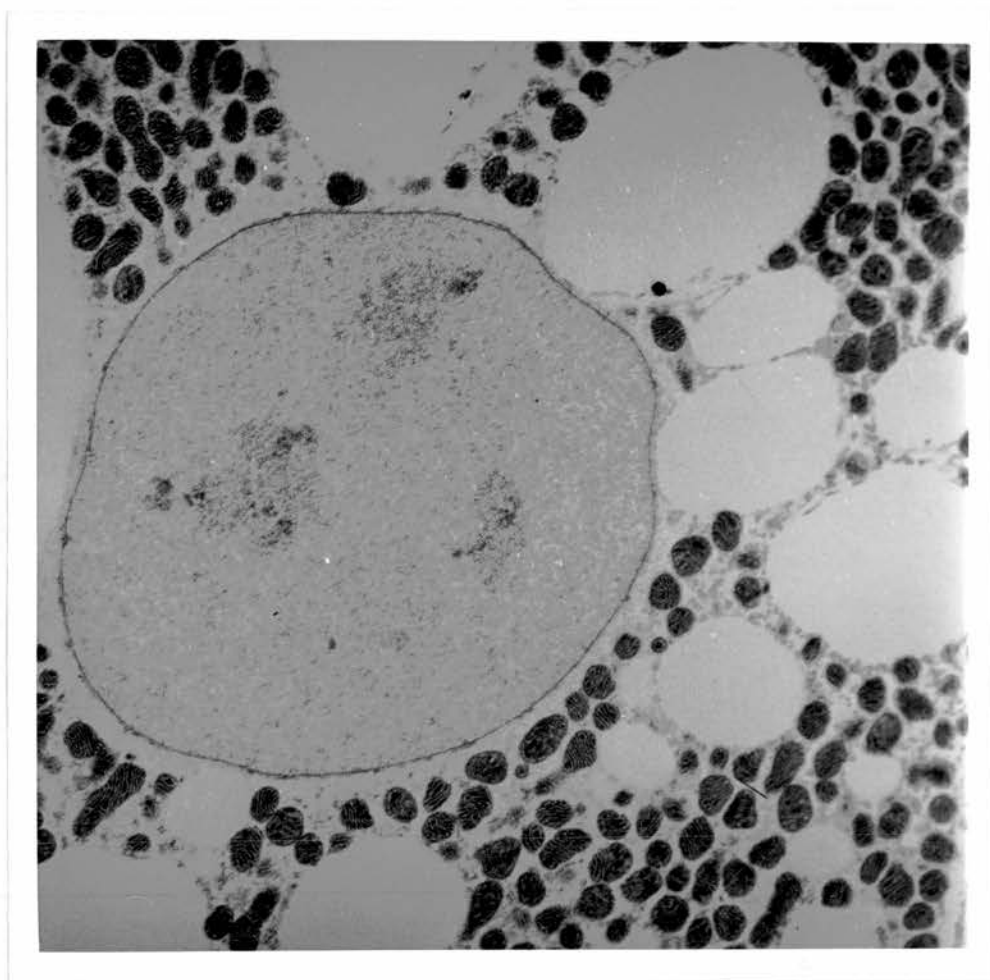


Fig. 5.4 Electron micrograph (X 12,090) of a cell from a dark area of perinephric fat from subject no. 26 showing numerous dense mitochondria and multilocular fat droplets.



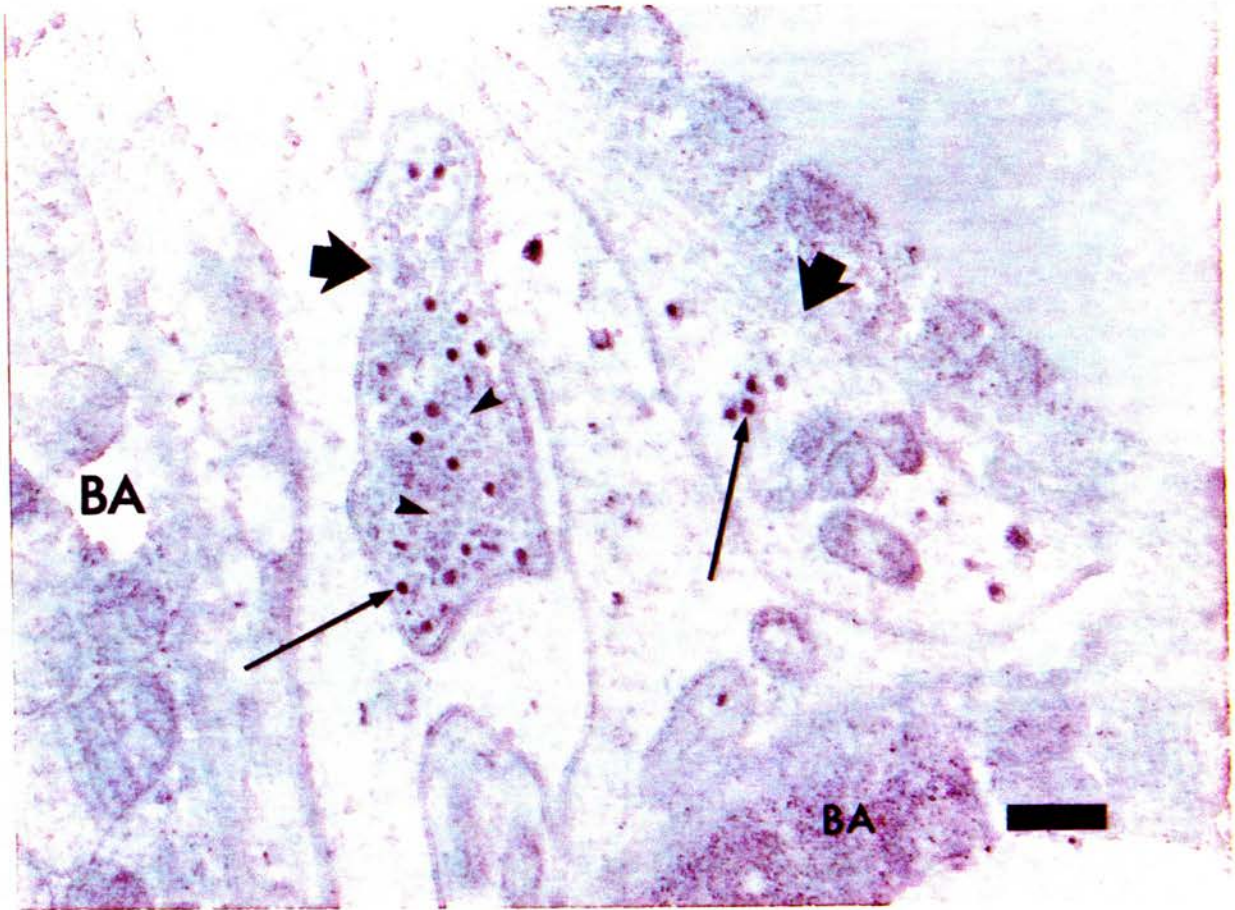


Fig. 5.5. Electron micrograph of brown adipose tissue obtained from subject no. 29.

A Schwann axon bundle in an intercellular space between brown adipocytes. The bundle contains axons (heavy arrows) exhibiting terminal features: large diameter (800-1000 Å) dense-cored vesicles (light arrows) and small diameter (400-600 Å) clear vesicles (arrowheads).

BA: peripheral cytoplasm of brown adipocytes  
Scale bar 0.5  $\mu\text{m}$  (X 26,000).

No.	Age (yrs)	Mitochondrial Cytochrome c Oxidase $\mu\text{mol 'O'}/\text{min}/\text{mg protein}$			Tissue Cytochrome c Oxidase $\mu\text{mol 'O'}/\text{min}/\text{g tissue}$			mg protein/g tissue		
		Light	Undiff	Dark	Light	Undiff	Dark	Light	Undiff	Dark
1	9	0.59	-	0.85	2.10	-	9.70	3.56	-	11.4
3	21	-	0.49	-	-	0.63	-	-	1.29	-
5	30	0.68	-	1.91	0.60	-	1.90	0.88	-	1.0
16	23	0.16	-	-	0.20	-	-	1.25	-	-
18	35	-	0.67	-	-	1.88	-	-	2.81	-
20	43	-	0.63	-	-	0.69	-	-	1.10	-
23	11	0.12	-	0.11	0.85	-	2.6	7.08	-	23.6
24	15	0.02	-	1.00	-	-	-	-	-	-
25	18	-	0.54	-	0.06	-	3.49	0.01	-	6.2
26	19	0.03	-	0.23	0.13	-	1.70	4.33	-	7.4
29	39	-	0.75	-	0.10	-	0.87	-	0.86	-
31	69	-	0.80	-	-	0.32	-	-	0.40	-
Warm Adapted Guinea Pig		6.42	-	-	39.9	-	-	6.21	-	-
Cold Adapted Guinea Pig		-	-	7.02	-	-	229	-	-	32.6

Table 5.2 Cytochrome c oxidase activity in tissue homogenates and mitochondria in 12 subjects compared to data from warm and cold adapted guinea pigs.

Undiff refers to tissue and mitochondria from areas undifferentiated into light and dark areas.



### 5.3.3. Uncoupling Protein

Mitochondrial density reflects non specifically the metabolic activity of the tissue under study whereas the presence of uncoupling protein is unique to brown adipose tissue mitochondria and thus indicative of the unique capacity for thermogenesis.

Values for 10  $\mu$ mol GDP binding are shown in Table 5.3. The range of all the human values both from light and dark areas lies midway between that obtained from warm and cold adapted guinea pigs indicating that the amount of uncoupling protein present is equivalent to that found in a partially cold adapted animal. In four out of the five subjects in whom macroscopic separation of light and dark areas for study were possible there was a tendency for a greater degree of GDP binding indicative of increased concentration of uncoupling protein in the dark areas. However, the results also demonstrate the presence of significant GDP binding in macroscopically "light" coloured areas demonstrating that active brown adipose tissue cannot be identified by visual inspection alone.

The presence of uncoupling protein would be of limited consequence if it were not functional i.e. capable of uncoupling respiration from oxidative phosphorylation in the brown adipocyte. As discussed earlier (Section 1.3.3.3) this protein confers on animal brown fat mitochondria a system of regulating proton conductance which is inhibited by purine nucleotide binding and activated by fatty acid. Hence the bioenergetic characteristics of the human mitochondria were investigated under the influence of these proton conductance regulators.

Age (yrs)	Subject No.	10 $\mu$ M GDP Binding nmol/mg protein		
		Light	Undifferentiated	Dark
9	1	0.165	-	0.174
11	23	0.140	-	0.150
15	25	0.058	-	0.125
19	24	0.082	-	0.298
19	2	-	0.160	-
21	3	-	0.120	-
30	5	-	0.378	-
34	28	0.210	-	0.160
WA-Guinea pig				0.572
CA-Guinea pig		0.056		

Table 5.3: Mitochondrial GDP binding: A comparison of human brown adipose mitochondria with warm adapted (WA) and cold adapted (CA) guinea pigs. Subject no. refers to Table 5.1. Undifferentiated refers to experiments in which pooled mitochondria from light and dark areas were studied.

#### 5.3.4. Bioenergetic Properties of the Mitochondria

Fig. 5.6 and Table 5.4 illustrate the respiratory and membrane potential measurements of mitochondria prepared from subject no. 14. The low basal membrane potential and high basal rate of respiration is indicative of the uncoupling of oxidative phosphorylation. The addition of albumin (Fig. 5.6), which binds fatty acids, thus resulted in an increase in membrane potential and slight inhibition of respiration measured by a decrease in slope. The addition of GDP resulted in a striking rise of membrane potential and 66% inhibition of respiration indicative of purine nucleotide binding preventing the dissipation of the proton electrochemical potential by the uncoupling protein.

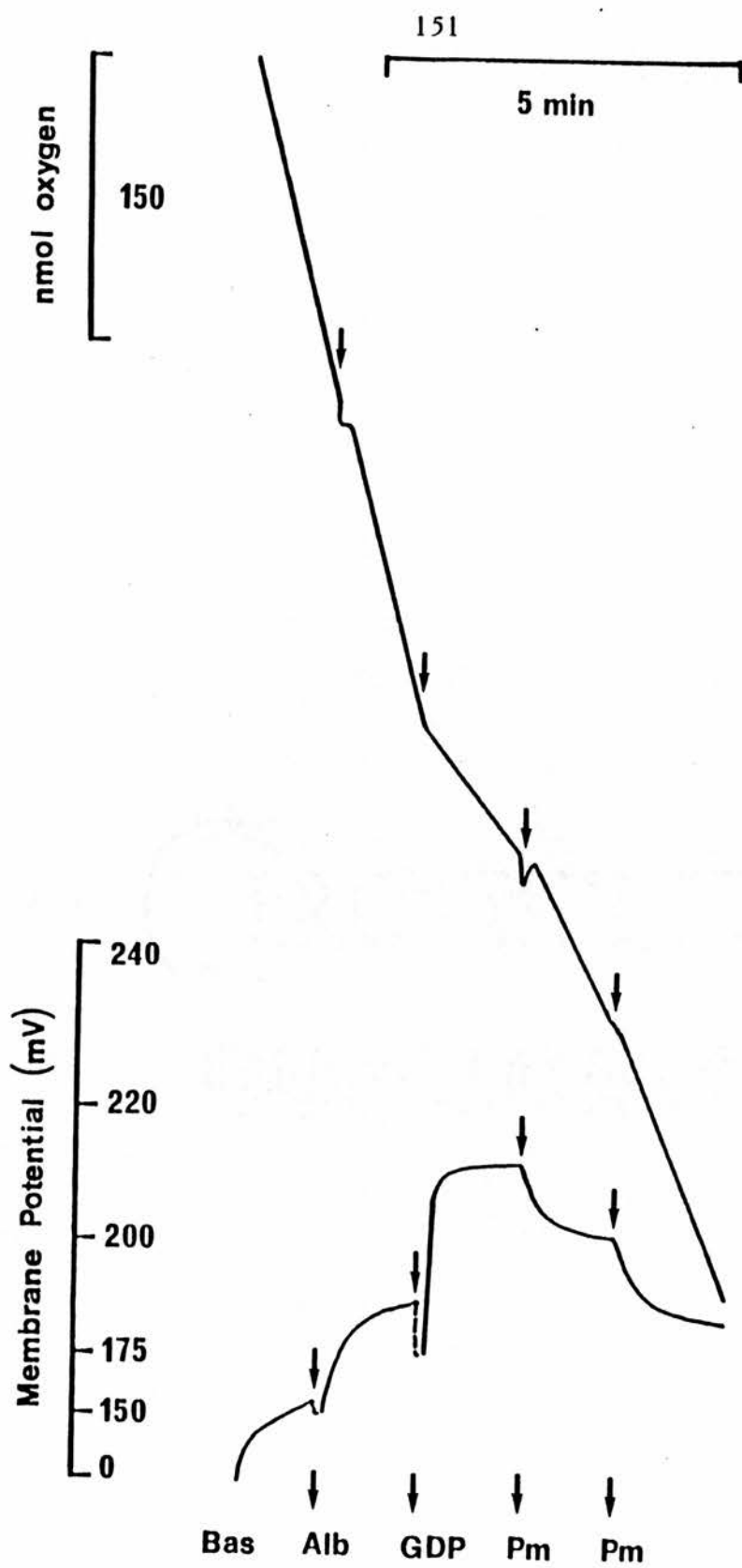
The subsequent addition of fatty acid in the form of palmitate overrides this inhibition resulting in a decrease in membrane potential and increase in respiration. This is attributable to the unmasking of the uncoupling protein proton shunt, mimicking the respiratory control seen in the warm and cold adapted guinea pig.

Table 5.4 also demonstrates that the basal respiratory rate and membrane potential of human brown adipose tissue mitochondria is intermediate to that of the warm and cold adapted guinea pig and provides further evidence for the partially cold adapted state of adult man.

Thus far, the presence of functional uncoupling protein has been demonstrated in human perirenal fat mitochondria. However, before human brown adipose tissue can be implicated in regulatory thermogenesis it is necessary to demonstrate uncoupling at the cellular level by the functional presence of  $\beta$ -adrenoceptors capable of activating the sequence of events shown in Fig. 1.3 following interaction with noradrenaline.



Fig. 5.6 (overleaf)



	Warm-adapted guinea pigs	Human	Cold-adapted guinea pigs
Respiration (nmol $^1\text{O}'$ /min/mg protein)			
Control	149	258	537
+ GDP	50	93	35
+ GDP + palmitate	73	159	132
Membrane Potential (mV/mg protein)			
Control	209	188	125
+ GDP	222	214	228
+ GDP + palmitate	211	188	203

Table 5.4: Bioenergetic properties of mitochondria prepared from human perinephric fat (subject no. 14) and from the dorsal brown fat pad of warm adapted and cold adapted guinea pigs. Respiration and membrane potentials of subject no. 14 are illustrated in Fig. 5.6 from which these data were derived.

### 5.3.6. Thermogenesis in isolated human brown adipocytes

The preparation of isolated brown adipocytes was attempted on several occasions but great difficulty was encountered in isolating functional intact cells using the same protocol for guinea pig adipocytes. This was mainly due to the comparatively small volume of tissue available, the low adipocyte yield following collagenase digestion and washing and the tendency for the human cells to lyse.

However, isolated adipocytes were successfully prepared from one subject (no. 13), a 3 year old male and these were stimulated with noradrenaline as described in Section 5.2.8.1.

Fig. 5.7 compares the catecholamine response of brown adipocytes prepared from warm and cold adapted guinea pigs with those obtained from this subject.

The addition of noradrenaline resulted in a four fold increment of the basal respiration of human brown fat cells. This compares to a six fold stimulation observed in the adipocytes of a warm adapted guinea pig and a 13 fold stimulation in those of a cold adapted animal. Therefore under maximal noradrenaline stimulation the respiratory capacity of the human brown adipocytes from this single subject approaches only 40% of the rates monitored for warm adapted guinea pig cells. However, these cells were capable of expressing a greater percentage of their cytochrome oxidase activity under noradrenaline stimulation (Fig. 5.7) and this probably reflects the lower mitochondrial complement of the human adipocyte which nonetheless contain a greater amount of uncoupling protein per mitochondrion (Section 5.3.2 and 5.3.3).

When this increase in respiration is expressed as a percentage of the cytochrome oxidase activity the human cells demonstrate a value of 7% once again, intermediate between that of warm adapted (1.2%) and cold adapted

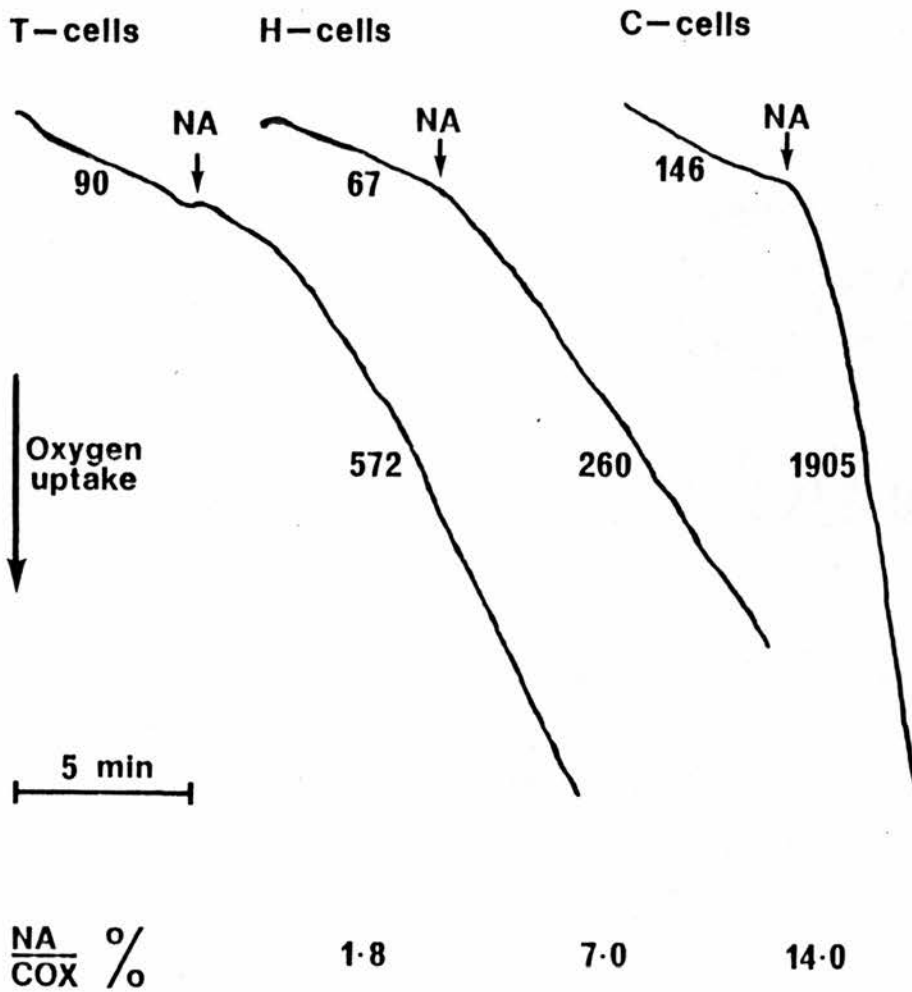


Fig. 5.7 Noradrenaline sensitivity of human brown fat cells (H-cells) prepared from subject no. 13 and comparison with the response of brown adipocytes from thermoneutral (T-cells) and cold adapted (C-cells) guinea pigs. Respiration was measured in oxygen electrode as described in Section 5.2.6. NA = addition of 10  $\mu\text{mol/l}$  noradrenaline. Basal and stimulated respiratory rates are shown against the traces in  $\text{nmol O' / min / } 10^5 \text{ cells}$ .

$\frac{\text{NA}}{\text{COX}} \%$  - noradrenaline induced stimulation of respiration expressed as a percentage of the cytochrome (oxidase) activity (COX) of each preparation.



(15%) guinea pig.

### 5.3.7. Total Tissue Respiratory Capacity in Response to Noradrenaline

#### 5.3.7.1. In vitro

From the GDP binding studies, human brown adipocytes appear to be in a partially cold adapted state - the value of GDP binding lying intermediate between that of the warm and cold adapted guinea pig (Section 5.3.3). As discussed above (Section 5.3.6) the percentage cytochrome oxidase activity expressed subsequent to noradrenaline stimulation lies in this intermediate range - the value from our single subject being 7%. Thus it can be assumed from these two independent assessments that at most 7% of the cytochrome oxidase activity would be expressed during stimulation. Using this assumption and a knowledge of the cytochrome oxidase activity and the total weight of perinephric fat surrounding one kidney the total respiratory capacity of this tissue can be calculated. A sample calculation is illustrated below for subject no. 2.

1. Total weight of perirenal fat	= 71.0 g
Weight of light area	= 58.9 g
Weight of dark area	= 12.1 g
2. Cytochrome C oxidase activity (Cox/g)	
Light area	= 0.13 $\mu\text{mol O/min/g}$
Dark area	= 1.70 $\mu\text{mol O/min/g}$

## 3. Cytochrome C oxidase activity/kidney

$$\begin{aligned}
 \text{activity} &= (\text{wt} \times \text{Cox/g}) \text{ light area} + \\
 &\quad (\text{wt} \times \text{Cox/g}) \text{ dark area} \\
 &= (58.9 \times 0.13) + 12.1 \times 1.70 \\
 &= 28.2 \text{ } \mu\text{mol O/min/kidney}
 \end{aligned}$$

## 4. Respiratory capacity induced by noradrenaline/2 kidneys

$$\begin{aligned}
 &= 7.0\% \text{ of } 28.2 \text{ } \mu\text{mol/min/kidney} \\
 &= 2.0 \text{ } \mu\text{mol O/min/kidney} \\
 &= 4.0 \text{ } \mu\text{mol O/min/kidney}
 \end{aligned}$$

5. Conversion of  $\mu\text{mol O}$  to  $\mu\text{l O}_2$ 

1  $\mu\text{mol}$  of  $\text{O}_2$  occupies 22.4  $\mu\text{l}$  at S.T.P.

$$\begin{aligned}
 1 \text{ } \mu\text{mol pf } \text{O}_2/\text{min/depot} &= 11.4 \times 4 \\
 &= 44.8 \text{ } \mu\text{l O}_2/\text{min/depot}.
 \end{aligned}$$

This calculation overestimates the values for two reasons. Firstly light areas with their lower GDP binding and mitochondrial complement will express a lower percentage of cytochrome oxidase activity than 7% which is that extrapolated for dark areas. This figure only applies to the 12.1 g of dark tissue - a lower value would be obtained by the remaining 58.9 g in this example. Secondly the value of 7% is obtained from a 3 year old child whose capacity for thermogenesis might be expected to be greater than that of an adult.

#### 5.3.7.2. In vivo

In the eight normal fasting subjects prior to noradrenaline infusion (Section 3.2.1.2) the basal oxygen consumption measured by indirect calorimetry ranged from  $173 \pm 219$  ml/min. During the final 15 minute period of the 45 minute noradrenaline infusion at a rate of  $0.1 \mu\text{g/kg IBW/minute}$ , this rose by 14 to 49 ml/min with a median rise of 31 ml/min.

#### 5.3.8. Comparison of the in vitro and in vivo Responses

The value of a rise in oxygen consumption of 31 ml/min in vivo in response to noradrenaline infusion which at most would achieve a plasma noradrenaline concentration of  $10^{-8}\text{M}$  compares to the mean estimated contribution of total perinephric adipose tissue of  $30 \mu\text{l O}_2/\text{min}$  following in vitro maximal stimulation at a noradrenaline concentration of  $10^{-5}\text{M}$  (Table 5.5). Thus using this method of calculation less than 0.1% of the thermogenic response to infused noradrenaline is accounted for by this tissue. Even if the best response -  $96 \mu\text{l O}_2/\text{min}$  in subject no. 1 is considered and these cells were in a maximally cold adapted state and expressed 15% of the activity during noradrenaline stimulation this value would still only be in the order of  $164 \mu\text{l O}_2/\text{min}$  - less than 1%.

### 5.4. DISCUSSION

The present study has considerably extended the current knowledge of brown adipose tissue activity in man.

Brown adipose tissue was identified in the perinephric adipose tissue of most adult subjects below, but less frequently above the age of 50 years. This is a similar pattern to that described by Heaton (1972) who consistently demonstrated multilocular adipocytes in the perirenal fat of subjects under

Estimated contribution to respiration  
in vitro of total perinephric fat  
( $\mu\text{l}/\text{O}_2/\text{min}$ )

Cytochrome C Oxidase Activity

$\mu\text{mol 'O'}/\text{min per kidney}$

$\mu\text{mol 'O'}/\text{min/g tissue}$   
Light Dark

Subject No.

Age  
(yrs)

9	1	2.10	9.70	60.1	96
11	23	0.85	2.60	15.2	24
15	24	0.79	3.90	17.6	28
18	25	0.06	3.49	15.0	12
19	26	0.13	1.70	28.2	44.8
21	3	0.63	-	6.9	11
25	27	0.56	0.57	33.7	53
28	4	0.10	0.50	5.8	9
30	5	0.60	1.90	16.8	26
34	28	0.78	10.00	39.7	62
39	29	0.10	0.87	13.1	21
46	6	0.10	0.50	5.8	9
51	30	0.33	1.10	34.7	54
52	7	0.09	-	22.5	35
59	8	0.19	-	6.3	10
64	9	0.13	1.30	8.8	14
69	31	0.32	-	22.4	35
74	10	0.10	-	10.3	16

Median 30  
Range 3-96  
 $\mu\text{l O}_2/\text{min}$

Table 5.5: Capacity of human perinephric fat for thermogenesis *in vivo*. It is assumed that human adipocytes express 7% of their total cytochrome C oxidase during noradrenaline stimulation (Fig 5.7; Section 5.3.7).

the age of 20 years, but only in 60-80% of subjects aged 21 to 60 years.

Light and electron microscopy demonstrated the presence of typical multilocular adipocytes with a high mitochondrial complement and for the first time catecholaminergic innervation, which has previously only been assumed, has been demonstrated in this tissue. Although the general trend was of a diminution of cytochrome c oxidase activity and GDP binding (indicative of the thermogenic capacity) with increasing age, there was no significant correlation between these variables in the small sample tested.

In all samples tested the presence of uncoupling protein was demonstrated by GDP binding. The values ranged from 0.378 nmol/mg protein to 0.082 nmol/mg protein. Recently Lean et. al (1986) using a solid phase radioimmunoassay (employing a specific rabbit anti-human uncoupling protein anti-serum) measured similar concentrations of uncoupling protein in perirenal and axillary adipose tissue in infants, children and adults. The range of values obtained from 17 adults aged 18 to 86 years was 30 µg/mg mitochondrial protein (0.468 nmol/mg) in a 24 year old subject to <0.8 µg/mg (0.125 nmol/mg) - the lower limit of their assay. Although axillary fat tended to have a higher concentration of uncoupling protein than perirenal adipose tissue, the highest value in normal subjects was obtained from perirenal tissue.

The work of Huttunen et. al. (1981) comparing the cytochrome c oxidase activity of brown adipose tissue in 7 indoor and outdoor workers which suggested increased activity of this enzyme in the latter group was interpreted as evidence of regulatory cold adaptation in man. However the cytochrome c oxidase activity of these subjects (0.047 to 0.025 µmol

$O_2$ /min/g tissue) falls well below the range of cytochrome c oxidase activity obtained in the present study (10.0 to 0.1  $\mu$ mol  $O$ /min/g tissue). This probably reflects differences in the method and delay of assay of the tissue but nonetheless demonstrates the hazards of extrapolating from a small number of subjects and inadequate control group.

The other major stimulus to brown adipose tissue metabolism that has been examined in man is that of catecholamines. These can be considered in two groups. Firstly, those studies in which the effects of the catecholamine secreting tumour phaeochromocytoma on brown adipose tissue activity have been examined, and secondly studies on the effects of sympathomimetic agents such as ephedrine and noradrenaline on thermogenesis from this tissue.

The association of "hibernomas" or "brown fat tumours" with phaeochromocytoma has already been discussed (Section 1.5.2.1) but the paucity of case reports suggests that this is an unusual finding. In the two cases presented in this study there was no evidence of these discrete, vascular brown fat deposits at laparotomy.

Ricquier et. al. (1982), using the same assay system employed in the present study, measured the cytochrome c oxidase activity in "dark" perirenal and periadrenal adipose tissue stained from two subjects with phaeochromocytoma, and compared this to white adipose tissue obtained from the omentum in the same subjects and also perirenal adipose tissue from one subject without phaeochromocytoma. As in the present study, they found cytochrome c oxidase activity and mitochondrial protein was greater in dark areas compared to light areas. Comparison with 'control' white adipose tissue suggested greater cytochrome c oxidase activity in adipose tissue from patients with phaeochromocytoma but the maximum value from peri-tumoural fat (0.37  $\mu$ mol  $O$ /min/g tissue) falls within the range obtained in subjects without phaeochromocytoma in the present study (0.09 - 9.7  $\mu$ mol

0/min/g tissue). This group had similar difficulties to ourselves in obtaining sufficient mitochondrial yields from the small amounts of tissue available at operation and only in one subject out of three was there a sufficient yield to perform a GDP binding assay. The value obtained from this subject, using a similar assay, was 0.21 nmol/mg protein which again falls within the range of GDP binding obtained from subjects without phaeochromocytoma in the present study (0.058 - 0.378 nmol/mg protein).

Recently, Lean et. al. (1986), using the radioimmunoassay method, have demonstrated higher concentrations of uncoupling protein in three subjects with phaeochromocytoma (40 year old female, 24  $\mu\text{g}/\text{mg}$  (0.375 nmol/mg); 21 year old male, 50  $\mu\text{g}/\text{mg}$  (0.781 nmol/mg) and a 22 year old female, 26  $\mu\text{g}/\text{mg}$  (0.406 nmol/mg). However, two subjects without phaeochromocytoma in this study had concentrations of uncoupling protein of 25 and 30  $\mu\text{g}/\text{mg}$  respectively. There is still not enough information in the literature to comment categorically, firstly whether these concentrations of uncoupling protein are significantly raised and secondly if they are the result of chronic noradrenaline stimulation. Thus, although noradrenaline appears to be the major factor controlling the concentration of uncoupling protein in rats (Mory et. al., 1984) this remains to be conclusively demonstrated in man.

The function in vitro of isolated human brown adipose tissue mitochondria and adipocytes in response to physiological stimulators (non-esterified fatty acids and noradrenaline) and inhibitors (GDP) has not been previously reported and confirms for the first time that the major assumption of equating the presence of uncoupling protein with a potential for thermogenesis in adult man is in fact correct.

The major question posed by this study was the contribution this tissue could make to the in vivo thermogenic response to noradrenaline as without an estimate of the magnitude of this parameter, comparison with results from animal models and extrapolation from histological and biochemical evidence for the presence of brown adipose tissue in man is of limited value. The inaccessibility of this tissue for study in vivo has proved a major problem in answering this fundamental question and only one other study to date has attempted this estimation (Astrup et. al., 1985b).

Fig. 5.8 illustrates the contribution of brown adipose tissue to in vivo sympathetic thermogenesis from the data of the present study and those of Astrup et. al. (1985b) in man with those obtained from the rat. This comparison demonstrates from the results of these two studies the minor importance of this tissue in sympathetic induced thermogenesis in man.

Astrup et. al. (1985b) tackled the problem of estimating the in vivo thermogenic response of brown adipose tissue to sympathetic stimulation in man using a totally independent method by measuring the increase in oxygen consumption following the ingestion of 1 mg/kg of the sympathomimetic agent, ephedrine, in normal subjects.

Perirenal brown adipose tissue (BAT) thermogenesis was calculated from the formula:

$$\text{BAT Thermogenesis} = (T_1 - T_c) \times \text{BAT Blood Flow} \times 3.68 \text{ (J/min)100 g)}$$

where  $T_1$  = local BAT temperature ( $^{\circ}\text{C}$ )

$T_c$  - core temperature ( $^{\circ}\text{C}$ )

The blood flow in perirenal adipose tissue was measured by a xenon clearance technique and local brown adipose tissue temperature by a flexible thermoprobe inserted in the perirenal tissue under ultrasound guidance. The mean peak percentage rise in oxygen consumption following ephedrine ingestion was 19% yet four out of the five subjects failed to demonstrate a



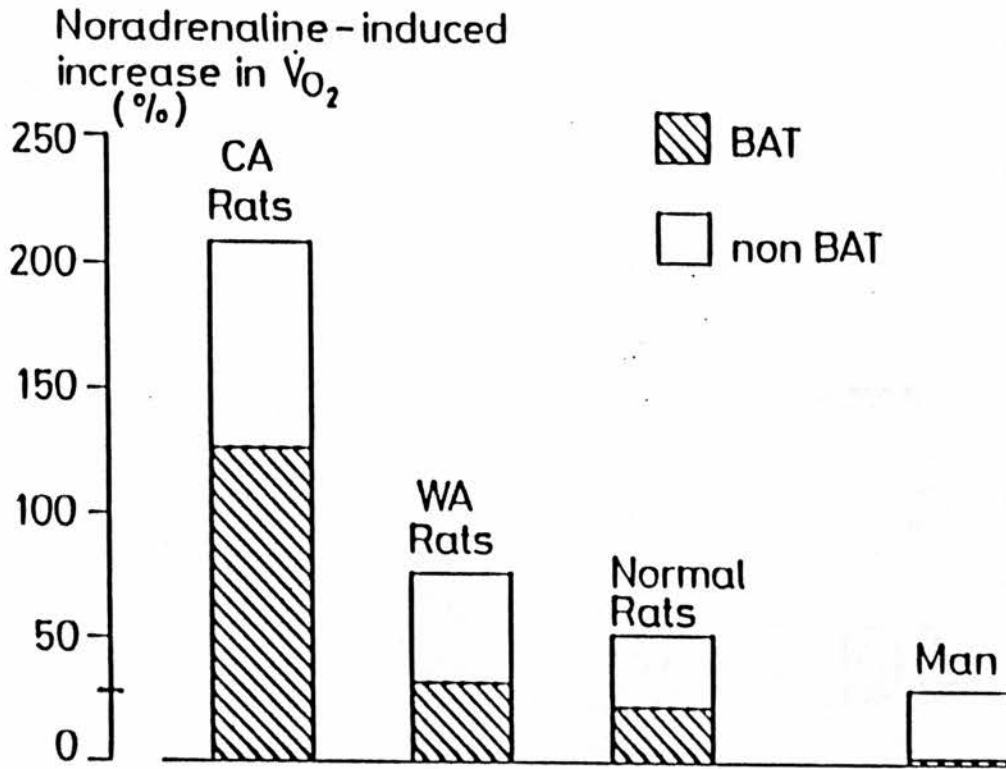


Fig. 5.8 The contribution of brown adipose tissue (BAT) and other tissues (non BAT) to noradrenaline induced thermogenesis in cold acclimatised (CA), warm acclimatised (WA), normal rats and adult man. The calculations are based on the results of Foster and Frydman (1978a), Rothwell and Stock (1979), Astrup et. al. (1985b) and the present data (Section 5.3.8)

Reproduced with permission from Astrup (1986)

significant rise in brown adipose thermogenesis. The fifth subject demonstrated an increase in oxygen consumption of  $80 \mu\text{l O}_2/\text{min}$  per 10 g of tissue - in the same order of magnitude as the total in vivo contribution of perirenal fat to the increase in oxygen consumption in the present study (range 4 -  $96 \mu\text{l O}_2/\text{min}$ ). Thus the only two assessments to date of the thermogenic activity of brown adipose tissue to sympathetic stimulation in living man have independently assessed the contribution of this tissue to the total response to be in the region of less than 1%.

Can these results be explained by an underestimation of the total brown adipose tissue depot in man as Lean et. al. (1986) have suggested that other sites may be important sources of adipocytes containing uncoupling protein? If the response of rats is to be extrapolated to man then the 1% contribution of perirenal tissue means this site represents only one fiftieth of the total brown fat deposit in man. This would result in the assumption of a brown adipose tissue depot in excess of 1 kg expressing maximal thermogenic activity and therefore containing a significant proportion of 'dark' tissue. Post mortem evidence from the present study, and those of Astrup et. al. (1985b) and Heaton (1972) does not support the presence of brown adipose tissue depot in man of these proportions.

If the noradrenaline stimulated increase in whole body oxygen consumption is not derived from brown adipose tissue in the human adult which tissue or tissues are then responsible for this use in metabolic rate?

In the study referred to above in which perirenal brown adipose tissue thermogenesis following oral ephedrine was measured (Astrup et. al. 1985b), oxygen consumption and blood flow was simultaneously measured in leg skeletal muscle. The increase in leg skeletal muscle oxygen consumption was

estimated to be  $68.2 \mu\text{m O}_2/\text{min}/100 \text{ ml}$  and assuming 50% of total body weight is skeletal muscle, the contribution of this tissue can be calculated to be 20-30 ml/min - compared to the whole body peak increase in response to ephedrine of 50 ml/min. Thus following ephedrine administration 50% the response can be accounted for by skeletal muscle thermogenesis.

The mechanisms contributing to this thermogenic response in muscle remain to be determined but may be related to blood flow (Mejsnar and Pacha, 1983) and catecholamine effects on calcium (Rasmussen and Clausen, 1982) and sodium pumping (Kjeldsen et. al., 1984) and protein synthesis (Emery et. al., 1984).

In conclusion although the present studies have confirmed and demonstrated the presence of functional brown adipose tissue in adult man with some potential for thermogenesis, the respiratory inadequacy of human brown fat suggests that considerable caution must be exercised in extrapolating the results of animal studies to the human. Based on this evidence, the reported thermogenic defects in Type I diabetic (Leslie et. al., 1986; Section 3.3.6), obese (Jung et. al., 1979) and lactating subjects (Illingworth et. al., 1986) are unlikely to be the result of defective brown adipose tissue metabolism in man.

Chapter VI

Conclusions and Perspectives

## CONCLUSIONS AND PERSPECTIVES

In this final chapter I will attempt to draw together the various strands of the arguments presented in this thesis to formulate how the present work has contributed to the current concepts of the control of energy balance and, in the light of this discussion, what direction future studies should follow.

The starting point of any discussion on the control of energy balance is the law of mass action which, in this context, states that the maintenance of a stable body weight is the result of the balanced equation shown below:

$$\text{ENERGY INPUT} \rightleftharpoons \text{ENERGY EXPENDITURE}$$

The balance of this equation can be disturbed in favour of weight gain by three methods:

i) Hyperphagia  $\rightarrow$   $\uparrow$  Energy Input  $\rightleftharpoons$  Energy Expenditure (constant)

ii) Energy Input (constant)  $\rightleftharpoons$  Energy Expenditure  $\downarrow$

or a combination of the above

iii) Hyperphagia  $\rightarrow$   $\uparrow$  Energy Input  $\rightleftharpoons$  Energy Expenditure  $\downarrow$

The present work has not formally addressed the difficult problem of assessing the contribution of hyperphagia to energy balance but the experiments in which energy expenditure was measured before and during

fat supplementation (Section 3.3.3.2) do demonstrate that in the short term caloric excess does not result in a measurable compensatory increase in energy expenditure.

Evidence in favour of the hypothesis shown in equation (ii) was presented in the experiments where the fall in RMR in Type I diabetic subjects with improvement in glycaemic control was associated with an increase in weight (Section 3.3.2). This does not necessarily infer a causal effect as the contribution of energy intake over that period was largely uncontrolled but does for the first time demonstrate an association between an alteration in energy expenditure and body weight.

I have demonstrated abnormal noradrenergic thermogenesis in Type I diabetes which appears to be independent of the degree of glycaemic control (Section 3.3.6). Type II diabetic subjects with similar levels of glycaemic control to those Type I diabetic subjects on CSII had normal thermic responses to noradrenaline (Section 4.3.6). Further, Illingworth et. al. (1986) have also demonstrated blunted thermic responses to noradrenaline in non-diabetic lactating subjects. These data can be interpreted as failing to support the hypothesis, extrapolated from animal models (Rothwell and Stock, 1981) that insulin is a major factor in determining sympathetic induced thermogenesis in man.

The conflicting data on noradrenergic responsiveness in obesity (Section 3.4.4) may be reconciled with recent data from Connacher et. al. (1987, in press) who examined the thermic responses to noradrenaline infusion in a large group of subjects (40 obese; 19 lean) and demonstrated that although a blunted thermic response to noradrenaline was more commonly associated with obesity than leanness, only 26% of obese subjects demonstrated a significantly blunted response. In the light of this data it may be more correct to interpret a diminished thermic response to

noradrenaline in terms of a "marker" or "risk factor" rather than an aetiological prerequisite to the obese state.

One can make an analogy with another common disorder, hypertension, which similarly has an incompletely understood aetiology. A minority of hypertensive subjects may have an identifiable cause such as a renal or endocrinological disorder but the vast majority are of multifactorial origin with genetic factors, cigarette smoking, obesity and abnormal vascular reactivity all independently contributing to the expression of the hypertensive state (Semple and Lever, 1986). This lack of understanding of the basic pathophysiology has however not resulted in therapeutic nihilism but has stimulated attempts to manipulate vascular tone (diuretics, beta blockers and calcium antagonists) and the hormonal milieu (angiotensin converting enzyme inhibitors) which although not proven to have an aetiological role nevertheless provide a means of altering the hypertensive state.

Similarly with obesity - although an abnormality of energy expenditure may not be involved aetiologicaly in the majority of cases, dietary manipulation and treatment with thermogenic agents may nevertheless provide us with a method of tackling this major problem. Ephedrine (Astrup et. al., 1985a) and more recently the new generation of  $\beta$ -agonists (Zed et. al., 1985) have been shown to facilitate weight loss in obese subjects and although the present work demonstrates that these are unlikely to be acting via perirenal brown adipose tissue, as the results of animal models would have led to speculate, nevertheless they may represent an important advance in the clinical management of the obese subject.

How then are these drugs acting and how may we study them? Substrate cycling (Section 1.3.1) is a much discussed method of achieving the dissipation of excess calories through futile interconversion of carbohydrate, fat or protein moieties. As discussed earlier the triglyceride-fatty acid cycle

has been studied in human adipose tissue (Hammond and Johnston, 1987, in press) and would appear to be energetically insignificant, thus focussing attention on muscle (Astrup et. al. (1985b) and liver (Berry et. al., 1985). The contribution of these metabolic masses to daily energy expenditure has to date only been crudely assessed when the RMR is expressed in terms of lean body mass in lean and obese subjects. However, results to date (Pittet et. al., 1976; Ravussin et. al., 1983) demonstrate that obese subjects do not appear to have a significantly lower RMR per kg lean body mass. With the increasing sophistication of in vitro techniques for studying the fate of radiolabelled substrates in human hepatocytes and skeletal muscle preparations and the advent of newer technology such as nuclear magnetic resonance which may permit the study of these processes in vivo and enable the contribution of substrate cycling to energy expenditure to be reexamined. Further, the study of the control of these processes may provide us with a new generation of pharmacological agents capable of accelerating energetically wasteful reactions with potential anti-obesity applications.

To conclude, the term, obesity, is simply a clinically useful description of those subjects who are greater than 120% ideal body weight. It is however, not a distinctly defined syndrome but probably represents the sum of a heterogeneous group of disorders which themselves may be multifactorial in origin. The spectrum of abnormalities predisposing an individual to obesity may range from simple gluttony to multiple, and as yet largely undefined, abnormalities of energy expenditure, energy intake and neuro-endocrinological disorders. The key lies in defining these various clinical entities more precisely.

The present studies have attempted to define more clearly the contribution of two factors in the control of energy balance, namely those of insulin and brown adipose tissue in man, and have demonstrated the



complexity and difficulties that may be encountered in extrapolating from animal models to adult man.

Ultimately, our understanding of the pathological disorders of under- and overnutrition will develop from a greater knowledge of the homeostatic mechanisms that have, for example, maintained my own body weight at  $69.5 \pm 1$  kg during the three year period that this work has been performed.

Epilogue

"Man is not a rat"

- Attributed to T.H. Huxley (1825-1895)

"The proper study of mankind is man"

- Alexander Pope (1773)  
An Essay on Man

Epistle i.

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### Publications

The following abstracts, papers and review articles arising from the work presented in this thesis have been published or are currently in press.

1. Leslie PJ, Cunningham S, Nicholls DG, Jung RT (1985)  
The energetics of brown fat in man.  
Scottish Medical Journal 30, 57 (Abstract).
2. Cunningham S, Leslie PJ, Hopwood D, Illingworth P, Jung RT, Nicholls D, Rafael E (1985)  
The characterisation and energetic potential of brown adipose tissue in man.  
Clinical Science 69, 343-348.
3. Lever JD, Nnodim J, Leslie PJ, Symons D (1986)  
Demonstration of catecholaminergic innervation of human perirenal brown adipose tissue at various ages in the adult.  
Anatomical Record 215, 251-255.
4. Leslie PJ, Jung RT, Isles TE, Baty J, Newton RW, Illingworth P (1986)  
The effect on energy expenditure of optimal glycaemic control using continuous subcutaneous insulin infusion (CSII) in Type I diabetic subjects.  
British Medical Journal 293, 1121-1126.
5. Leslie PJ, Jung RT, Newton RW, Isles TE, Baty J (1987)  
Energy expenditure of Type II diabetic subjects on metformin or sulphonylurea therapy.  
Clinical Science (in press).
6. Leslie PJ, Jung RT, Nicholls DG, Cunningham S, Isles TE (1987)  
Energy expenditure in Type I and Type II diabetes mellitus: a role for brown adipose tissue?  
Health Bulletin (in press).

READY.

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1 PRINT"*****"
2 PRINT"SHORT-TERM MEASUREMENT OF METABOLIC RATE"
3 PRINT"*****"
5 D=7:DIND(2531),DR(D),T(D),G(14):M=1000:L=M*4:T=10:C=M/T:MX=160
10 DEFFNM(MM)=INT(MM*M+.5)/M:DEFFNC(CC)=INT(CC*C+.5)/C
15 G$="PRESS <RETURN> TO CONTINUE":A$=LEFT$(G$,14)
20 B$=" ADJUST":C$="CALIBRATION PROCEDURE (CONT):"
25 RS$="PRE-CALIBRATION SEQUENCE:"
30 SF$=" * ENSURE SAMPLE FLOW ON CO2 METER READS APPROX. 1L/MIN"
35 CM$=" * ENSURE SAMPLE FLOW ON O2 METER IS NEAR '2' AND STEADY"
40 E$="INVALID OPTION (DURATION <"
50 IT$="INITIAL TIME":FT$="FINAL TIME"
60 O1$=" 1. TABULAR FORM
65 O2$=" 2. MEANS +- SEM
70 PRINT"SELECT BY NUMBER:"PRINT" 1. EXPERIMENT - DATA STORAGE
80 PRINT" 2. EXPERIMENT - NO STORAGE"
81 PRINT" 3. DATA RETRIEVAL"
82 PRINT" 4. CALCULATE BUILD-UP AND NORADRENALINE REQUIREMENTS"
90 GETH$:IFH$="3"GOTO6500
95 IF H$="4" THEN GOTO14000
100 IFH$="1"GOTO130
105 R$="RESULTS (SELECTABLE LIMITS):"
110 IFH$="2"GOTO160
120 GOTO90
130 PRINT"LOAD STORAGE DISKETTE INTO DRIVE 1":GOSUB3000
140 INPUT"FILE NAME":NA$
142 INPUT"SUBJECT NAME":D$
144 INPUT"DATE":T$
146 INPUT"BUILD UP/NORADR":U$
148 INPUT"HEIGHT":V$
150 INPUT"WEIGHT":W$
155 PRINT:GOSUB3000
160 PRINTLEFT$(C$,22):"
170 PRINT" * CHECK THAT 'SUPPLY' LIGHT IS ON"
172 PRINT" * CHECK THAT ANALYSERS HAVE BEEN OPERATING CONTINUOUSLY"
175 PRINT" * CHECK CONDITION OF DRYING AGENT"
180 PRINT" * SWITCH ON 'OPERATE' BUTTON TO ACTIVATE CALORIMETER PUMPS"
190 PRINT" * TURN GAS CONTROL VALVE TO 'NITROGEN GAS'"
210 GOSUB3500:GOSUB3000
220 IFCTTHENCH=0:GOSUB5500:GOTO540
250 GOSUB2510:PRINTC$
540 PRINT" * SET 202 SWITCH TO 1% RANGE":GOSUB3000
550 IFCTTHENCH=1:GOSUB8110:RD=RD+1:DR(RD)=S/T:PRINTRS$:GOTO650
560 GOSUB7500:PRINTC$
650 PRINT" * SET 202 SWITCH TO 25% RANGE"
670 PRINT" * TURN GAS CONTROL VALVE TO 'CO2 GAS'"
740 GOSUB3500:GOSUB3000:IFCTTHENCH=0:GOSUB5500:GOTO850
750 GOSUB2510:PRINTC$
850 PRINT" * TURN GAS CONTROL VALVE TO 'ROOM AIR':GOSUB 3500:GOSUB3000
880 IFCTTHENCH=1:GOSUB5500:GOTO1000
930 GOSUB2510:PRINT"MEASUREMENT PROCEDURE:"
1000 PRINT" * NOW CALORIMETER WILL MEASURE ROOM CO2 AND O2 LEVELS"
1020 IFCTTHENGOSUB3500:GOSUB3000:GOTO1080
1030 GOSUB3000:GOSUB2510:GOSUB7500
1080 IFCTTHENCH=0:GOSUB5500:PRINT" * TURN OFF 'OPERATE' SWITCH"
1090 IFCTTHENCH=1:GOSUB8110:DR(P)=S/T
1100 T(1)=D(1)*L:T(2)=D(2)*MX:T(3)=D(3)
1110 POKE158,0:INPUT"INPUT ROOM TEMPERATURE DEG.C.":T(3):SW=1
1120 IFCTTHENB=P:PC=P:GOSUB7000:GOTO1730
1130 D(3)=T(3)
1140 PRINT" * TURN GAS CONTROL VALVE TO 'SAMPLE'"
1150 PRINT" * POSITION HOOD FOR SUBJECT COMFORT &"
1160 PRINT" * B$"VENTILATED HOOD FLOW RATE TO"SPC(5)"45L/MIN"
1170 PRINT" * TO START - "A$:GOSUB3140:PRINT" *";
1210 D(4)=(9.993-.3592*D(3)+.03775*D(3)^2)*.4:IFCTTHEN1230
1220 B=4
1230 IN=C-D(1)-D(2):IFCTTHEN7110
1350 PRINT" * TO TERMINATE - "A$:GOSUB11220
1500 TA=TI:TB=TI-TB
1505 IFR=0THENTB=300
1510 IF(TI-TA)>(3599-TB)THENGOTO1530
1515 GETX$:IFX$=CHR$(13)OR R=360 THEN GOSUB4500:GOTO1700
1520 GOTO1510
1530 TB=TI:CH=0:FORJ=1TO3:GOSUB8110:CH=CH+1:NEXT
1540 D(B)=D(B)/4:D(B-1)=D(B-1)/MX:D(B-2)=D(B-2)/L
1545 R=R+1:PRINT" |":IFR<TTHENPRINTSPC(1)R:GOTO1555
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1547 IF R=>C THEN Q$=STR$(R):Q$=RIGHT$(Q$,LEN(Q$)-1):PRINT Q$":GOTO1555
1550 PRINTSPC(0)R;
1555 EN=C-D(B-2)-D(B-1):TR=(EN*D(2)/IN)-D(B-1)
1560 VS=.45*(D(B)-D(4))*273/((273+D(3))*760)
1570 Q2=VS*TR:CQ2=VS*(D(B-2)-D(1))
1575 RQ=CQ2/Q2:HF=16.494*Q2+4.628*CQ2
1580 QA=FNM(RQ):VA=FNM(HF)
1590 CP=FNM(CQ2):OC=FNM(Q2)
1595 IFCTTHEN1650
1600 PRINT"|"CP
1610 PRINTSPC(13)"|"OC:PRINTSPC(21)"|"";
1620 IFQA<1THENPRINT"||";
1630 PRINTQA:PRINTSPC(29)"|"VA:PRINTSPC(37)"|"
1650 B=B+4:D(B-3)=CQ2:D(B-2)=Q2:D(B-1)=RQ:D(B)=HF
1655 IFCTTHEN7170
1660 GOTO1500
1700 PRINTSPC(2)"EXPERIMENT TERMINATED"
1705 PRINT" * REMOVE HOOD"
1720 GOSUB 3000
1725 IF H$="1"THEN GOSUB 20000
1726 PRINT RS$:CT=1:PC=P:GOTO 190
1730 IFH$="1"GOTO2420
1740 IFSWTHENPRINTSPC(0)R$:SW=0:GOTO1750
1745 PRINTSPC(2)R$
1750 PRINTO1$:PRINTO2$
1760 PRINT" 3. PRINT OUT RESULTS
1765 PRINT" 4. TERMINATE"
1770 PRINT"SELECT BY NUMBER
1780 A=0:H=0:Q=0:Q2=0:V=0:V2=0
1800 GETM$:IFM$="1"THENPRINT"|"O1$:GOTO1940
1810 IFM$="2"THENPRINT"|"O2$:GOTO1940
1820 IF M$="4" THEN PRINT"DO YOU WISH TO STORE ANY COLLECTED MEANS":GOTO 1850
1830 IF M$="3"THEN PRINT"RESULTS BEING PRINTED":GOTO15000
1840 GOTO1800
1850 GET X$:IF X$<>"Y" AND X$<>"N" THEN 1850
1860 IF X$="N" THEN GOTO 12900
1865 GN$=NA$+"/GR"
1870 DOPEN#3,(GN$),D1,W
1871 IF DS<>0 THEN PRINT DS$:STOP
1880 FOR I=1 TO 14:PRINT#3,G(I):NEXT I
1881 IF DS<>0 THEN PRINTDS$:STOP
1890 CLOSE3:GOTO 12900
1940 IFHTHENPRINT"RE-ENTER LIMITS:
1950 INPUT"INITIAL TIME (MIN):|";X
1955 IFX=0THENPRINT"|"MID$(E$,1,14)"":H=1:GOTO1940
1960 IFX>RTHENPRINT"|"IT$"EXCEEDED BY"X-R"MIN":H=1:GOTO1940
1970 INPUT" FINAL TIME (MIN):|";Y
1980 IFY>RTHENPRINT"|"FT$"EXCEEDED BY"Y-R"MIN":H=1:GOTO1940
1990 IFX-Y>0THENPRINT"|"IT$"EXCEEDS "FT$"BY"X-Y"MIN":H=1:GOTO1940
2000 IFM$="1"GOTO2100
2005 PRINT"OK
2010 FORI=D*(X-1)+TTOD*(Y-1)+TSTEPD
2020 GOSUB6000:NEXT:W=Y-X+1:GOSUB10000:SW=1:GOTO1740
2100 GOSUB11220
2110 FORI=XTOY:F=I*D+4:CR=CR+1:SC=CR/16
2120 IFSC=INT(SC)THENPRINT"|"LEFT$(G$,18)"SCROLL":GOTO2140
2130 GOTO2150
2140 GETX$:IFX$=CHR$(13)THENI=I-1:PRINT"|"":GOSUB11220:NEXT
2145 GOTO2140
2150 PRINT"|"":IFI<TTHENPRINTSPC(1)X$:GOTO2170
2160 PRINTSPC(0)X;
2170 PRINT"|"FNM(D(F-3)):PRINTSPC(13)"|"FNM(D(F-2))
2180 PRINTSPC(21)"|"FNM(D(F-1)):PRINTSPC(29)"|"FNM(D(F))
2190 PRINTSPC(37)"|"
2200 X=X+1:NEXT:GOSUB4500:CR=0:GOTO1740
2420 PRINT"DATA STORAGE":D(0)=B

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2430 DOPEN#2,(NA$),D1,W
2431 IF DS<>0 THEN PRINTDS$:STOP
2432 FOR I=0 TO B:PRINT#2,D(I)
2433 IF DS<>0 THEN PRINT DS$:STOP
2434 NEXT:YY$=CHR$(13)
2435 PRINT#2,NA$;YY$;D$;YY$;T$;YY$;U$;YY$;V$;YY$;W$
2436 IF DS<>0 THEN PRINT DS$:STOP
2440 CLOSE2:PRINT"DATA STORED
2470 GOTO1740
2500 GOSUB3500:GOSUB3000
2510 GOSUB4100:GOSUB7500
2520 RETURN
3000 PRINTG$:POKE158,0
3140 GETX$:IFX$=CHR$(13)THENRETURN
3200 GOTO3140
3500 PRINTSF$:PRINTCM$:RETURN
4100 I$="000000":TX=TI
4105 IF P=4 OR PC=2 THEN RETURN
4130 DX=7200
4140 DY=TI:PRINT"SYSTEM FLUSH -"DX/60"SEC. DELAY PC=";PC;" P=";P
4150 IFP=0THENDY=P:GOTO4170
4160 DY=TI-DY
4170 IF(TI-TX)>(DX-DY-1)THENRETURN
4180 GOTO4170
4500 PRINT"□":FOR J=1TO38:PRINT"=";:NEXT:RETURN
5000 PRINT"□CALIBRATION PROCEDURE:□
5010 IFP>0THENPRINTSPC(21)"□ (CONT):□
5020 RETURN
5500 RD=RD+1:PC=PC-1
5510 GOSUB4100:GOSUB8110
5520 DR(RD)=S/T:PRINTRS$
5530 RETURN
6000 Q=Q+D(I):Q2=Q2+D(I)^2
6010 V=V+D(I+1):V2=V2+D(I+1)^2
6020 RETURN
6500 PRINT"LOAD DATA DISKETTE INTO DRIVE 1":GOSUB3000
6505 PRINT"DATA FILE FOR ACCESS IS : ";
6506 INPUT NA$
6510 DOPEN#2,(NA$),D1
6511 IF DS<>0 THEN PRINT DS$:STOP
6515 INPUT#2,D(0):B=D(0):R=(B-6)/D
6516 IF DS<>0 THEN PRINT DS$:STOP
6520 FORI=1TOB:INPUT#2,D(I):NEXT
6521 IF DS<>0 THEN PRINT DS$:STOP
6525 INPUT#2,N$,D$,T$,U$,V$,W$
6526 IF DS<>0 THEN PRINT DS$:STOP
6527 IF ST=64 THEN CLOSE2
6529 IF M$="3" THEN CMD5
6530 PRINT"FILE NAME: ";NA$:PRINT"SUBJECT NAME: ";D$
6535 PRINT"DATE: ";T$:PRINT"BUILD UP/NORADR: ";U$
6537 PRINT"HEIGHT: ";V$:PRINT"WEIGHT: ";W$
6540 PRINT"EXPERIMENT DURATION:"R"MIN":SW=1
6550 IF M$="3"THEN GOTO15020
6560 GOTO 1740
6570 END
7000 PRINT"PRE-CALCULATION IN PROGRESS□
7005 DR(D)=D(3)
7008 FOR I=1 TO R:G=0.80:LX=L*G:FA=I/R
7010 FORJ=1TO2:E=DR(J+2)-DR(J)
7020 T(J+3)=DR(J)*LX*FA/E
7030 IFI=RTHENT(J+5)=E:GOTO7050
7040 T(J+5)=LX-(LX-E)*FA
7050 D(J)=(T(J)-T(J+3))*G/T(J+5)
7060 G=20.94:LX=G*MX
7070 NEXTJ
7080 IFI=RTHEND(3)=DR(D):GOTO1210

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7090 D(3)=T(3)-(T(3)-DR(D))/#FA
7100 GOT01210
7110 G=0.80:JB=1:JC=3:LX=L*G:E=DR(JC)-DR(JB)
7120 FORJ=(B-1)TOB:XC=D(J)
7130 D(J)=(XC*LX/G)-T(JC+1))*G/T(JC+3)
7140 G=20.94:JB=2:JC=4:LX=G*MX
7150 NEXTJ
7160 B=B+1:GOT01555
7170 B=B+2
7180 NEXTI
7190 RETURN
7500 P=P+1:ONPGOSUB8000,8010,8040,8070,8110,8110
7510 CH=CH+1:IF P=2 OR P=4 THEN CH=0
7520 IFP=4GOT07550
7530 RETURN
7550 PRINT"END OF CALIBRATION":RETURN
8000 PRINT"CO2 ZERO %=":GOT08100
8010 GOSUB5000:PRINT" *B#"ZERO KNOB - LOCATED ABOVE
8020 PRINT"LEFT OF %O2 SWITCH - TO READ ZERO
8030 PRINT"O2 ZERO %=":GOT08100
8040 GOSUB5000:PRINT" *B#"CO2 SPAN ADJUSTMENT SCREW (BELOW ZERO KNOB) TO
8050 PRINT".800 USING SCREWDRIVER"
8060 PRINT"CO2 SPAN %=":GOT08100
8070 GOSUB5000:PRINT" *B#"SPAN - LOCATED ABOVE RIGHT
8080 PRINT"OF %O2 SWITCH - TO 20.94%
8090 PRINT"O2 SPAN % =
8100 PRINT"B#"UNTIL SATISFACTORY"SPC(15)G#:GOT09500
8110 B=B+1:S=0:FORI=1TOT:GOT09500
8120 S=S+N:NEXT:IFCTTHENRETURN
8130 D(B)=S/T:IFB>2THENRETURN
8140 IFCH=0THEND(1)=D(1)/L:PRINT"ROOM CO2% ="FNM(D(1)),:RETURN
8150 D(2)=D(2)/MX:PRINT"ROOM O2% ="FNC(D(2))":RETURN
8160 IFP=1THENPRINT":GOT08190
8170 PRINT"
8180 IFP=4GOT08230
8190 IFN>=L-2ANDN<=L+2THENPRINTSPC(13)"1.000":GOT09000
8200 IFABS(N)<40THENGOSUB12000:GOT09000
8210 N=FNM(N/L):IFABS(N)<1THENPRINTSPC(13)N""":GOT09000
8220 PRINTSPC(12)N""":GOT09000
8230 PRINTSPC(12)FNC(N/MX)""":GOT09000
9000 GETX$:IFX#=CHR$(13)THENRETURN
9500 POKE158,0:OPEN1,9,CH:GET#1,J#,K#:K=(ASC(K#)-224)
9510 IFK<0THENHI=(K+32)*-1
9520 IFK>=0THENHI=K
9530 HI=HI*256
9540 IFJ#=""THENLO=0:GOT09560
9550 LO=ASC(J#):IFK<0THENLO=LO*-1
9560 N=LO+HI:CLOSE1
9570 IF CH=0 THEN N=N*2
9580 IF P>4 GOT0 8120
9590 GOT08160
9600 OPEN5,4:CMD5
9605 POKE158,0
9610 FOR A=1 TO 7:PRINT DR(A):NEXT A:A=0
9615 FOR C=1 TO 18:PRINT D(C):NEXT C:C=0
9620 PRINT#5:CLOSE5
9630 GOSUB3000:RETURN
10000 U=FNM(Q/W):Z=FNM(V/W)
10030 IFY-X=0THENEQ=0:EV=0:E1=0:GOT010065
10040 E1=((V2-V+2/W)/(W-1))*.5
10050 EQ=FNM(((Q2-Q+2/W)/(W-1))*.5)
10060 EV=FNM(E1)
10065 IFATHENRETURN
10070 PRINT"MEAN RQ ="U"+--"EQ
10080 PRINT"MEAN HP (KJ/MIN) ="Z"+--"EV
10090 PRINTSPC(8)"(MJ/24H) ="FNM(V/W*1.44)+"--"FNM(E1*1.44)

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10100 REM SUBROUTINE TO COLLECT MEANS
10110 PRINT "DO YOU WISH TO COLLECT THIS MEAN?"
10120 GET Z$:IF Z$<>"Y" AND Z$<>"N" THEN 10120
10130 IF Z$="N" THEN GOTO 10900
10140 YA=YA+1:G(YA)=Z
10900 PRINT "DO YOU WISH TO INSERT A BLANK?"
10910 GET Y$:IF Y$<>"Y" AND Y$<>"N" THEN 10910
10920 IF Y$="N" THEN 11000
10930 Z=0:GOTO 10140
11000 RETURN
11215 OPEN 5,4:CMD5
11220 PRINT "
11230 PRINT " | TIME | CO2 | O2 | | H.P. |
11240 PRINT " | MIN | PROD. | CONS. | R.Q. | KJ/MIN |
11250 PRINT " |-----|-----|-----|-----|-----| " : RETURN
12000 N0=INT((ABS(N)-1/M)/4):P$=STR$(N0):P$=RIGHT$(P$,LEN(P$)-1)
12010 IF P=1 THEN PRINT " ";
12020 IF N<0 THEN PRINT SPC(13) "-.00" P$: RETURN
12030 PRINT SPC(13) ".00" P$: RETURN
12900 PRINT "THE END":GOTO 13000
13000 FOR I=1 TO L: NEXT: PRINT " ";: END
14000 PRINT "PUT IN IDEAL BODY WEIGHT(KG) OF SUBJECT"
14010 INPUT "W= " ; W
14020 V%=41*W*568.25/(1354*2):V=INT(V%)
14030 R=W*60/100
14040 PRINT "SUBJECT THEREFORE NEEDS " ; V ; "ML OF RECONSTITUTED BUILD-UP"
14050 PRINT "MAKE UP 0.5MG NORADRENALINE WITH 50ML OF SALINE"
14060 PRINT "PUMP SHOULD BE SET AT " ; R ; "ML/HR FOR THIS SUBJECT"
14070 END
15000 REM SUBROUTINE TO PRINT OUT RESULTS
15010 OPEN 5,4:GOTO 6529
15020 CMD5:GOSUB 11220
15025 PRINT#5:CLOSE 5
15030 OPEN 6,4,2
15035 OPEN 7,4,1
15040 PRINT#6," 999 .999- .999- 9.999- 9.999 "
15050 FOR I=1 TO R:F=I*D+4:
15060 PRINT#7,I:FNM(D(F-3));FNM(D(F-2));FNM(D(F-1));FNM(D(F))
15070 NEXT I
15080 CLOSE 7:CLOSE 6
15090 GOTO 1740
16100 END
20000 REM SAFETY STORAGE
20010 PRINT " SAFETY STORAGE IN PROGRESS
20020 NP$=NA$+"/S"
20025 B=B+2
20030 D(0)=B
22430 DOPEN#2,(NP$),D1,W
22431 IF DS<>0 THEN PRINT DS$:STOP
22432 FOR I=0 TO B:PRINT#2,D(I)
22433 IF DS<>0 THEN PRINT DS$:STOP
22434 NEXT:YY$=CHR$(13)
22435 PRINT#2,NA$:YY$:D$:YY$:T$:YY$:U$:YY$:V$:YY$:W$
22436 IF DS<>0 THEN PRINT DS$:STOP
22440 CLOSE 2:PRINT "DATA STORED
23000 RETURN
READY.

```